REMARKS

Status of the application

Claims 1-3, 6-7, 9-10, 14-17, 19, 27-28 and 32 are pending in the application, with claims 10, 14-15, 17 and 19 being withdrawn from consideration by the Examiner as directed to non-elected inventions. Claims 1-3, 6-7, 16, 19, 27-28 and 32 are under examination and stand rejected in the instant Office Action.

With entry of the present response, claims 1-3 have been amended to replace the recitation of "metabotropic glutamate disorder" with the phrase "drug dependence." Support for the amendment is replete in the specification, e.g., at pages 23-24. Dependent claims 6 and 7 have been accordingly amended to ensure proper antecedent basis. Claims 19 and 27 have been amended to delete the recitation of depression. Applicants submit that the claim amendments introduced herein are intended to more distinctly claim Applicants' invention or to improve clarity of claim language. They should not be construed as acquiescence of any ground of rejections.

Applicants additionally present the following remarks to clarify the various aspects of the non-obviousness nature of the claimed invention, the only substantive issue remaining in the prosecution of the subject patent application.

The prior art taught away from antagonizing mGluR2/3 in treating withdrawal symptoms

In rendering and maintaining the obviousness rejection of the subject invention, the Examiner relies on Adams et al. (USPN 6,407,094) for alleged teaching of the use of mGluR2/3 antagonists in treating addictive disorders. As clarified in Applicants' previous responses, this reference disclosed certain compounds that are purportedly antagonists of mGluR2/3. Adams et al. merely speculated about possible use of the compounds in the treatment of a variety of diseases or conditions, including addictive disorders. However, there were neither experimental data nor plausible substantiation in Adams et al. to suggest that the compounds are indeed effective to treating drug

dependence. More importantly, as explained below, the pure speculation of Adams et al. is contradictory to results from actual scientific studies that were published in peer reviewed journals.

The Examiner is advised that the present inventors were the first to experimentally demonstrate that a mGluR2/3 antagonist is effective in the treatment of drug withdrawal. The inventors' study was described in detail in Example 1 of the subject specification and also in a corresponding post-filing publication, Kenny et al., (J. Pharmacol. Exp. Ther. 306:1068-76, 2003; copy attached). Prior to the subject invention, the consensus view in the scientific community on the connection between modulating mGluR2/3 receptors and reducing withdrawal symptoms was the opposite to that reflected by the results obtained by the present inventors. At that time, several research groups have shown that agonists (not antagonists) of mGluR2/3 were able to attenuate withdrawal symptoms and to treat morphine or nicotine dependence. The apparent difference between the prior art studies and the subject disclosure is explained in the specification, e.g., at page 43, middle paragraph; and page 45, 3rd paragraph to page 46, 1st paragraph.

Specifically, Helton et al. (Neuropharmacol. 36:1522-6, 1997; copy attached) reported that mGluR2/3 agonist LY354740, when administered to nicotine dependent rats, resulted in a dose-dependent attenuation of the enhanced auditory startle response following withdrawal of nicotine. Helton et al. further noted that their data indicate that the mGluR2/3 agonist could be effective in treating nicotine withdrawal symptoms during smoking cessation in humans. Similar data suggesting the use of mGluR2/3 agonists in treating withdrawal symptoms were also reported in other scientific papers published prior to the subject invention. For example, Vandergriff and Rasmussen (Neuropharmacol. 38:217-22, 1999; copy attached) reported that mGluR2/3 agonist LY354740 was able to reduce symptoms in morphine dependent rats following withdrawal, and suggested its potential therapeutic use in treating human opiate dependence. In another study, Fundytus and Coderre (Brit. J. Pharmacol.

121:511-4, 1997; copy attached) reported that mGLuR2/3 agonist DCG-IV was able to significantly attenuate withdrawal symptoms in morphine dependent rats. The authors suggested that activation of the mGluR receptors could reduce withdrawal symptoms in human patients.

For the above, it is readily apparent that, prior to the subject invention, scientific publications in the prior art taught that activation of mGluR2/3 receptors could produce beneficial effects in treating drug dependence (e.g., reducing withdrawal symptoms). By extension, one would understand that inhibition of mGluR2/3 receptors (e.g., via an antagonist compound) is likely to exacerbate withdrawal symptoms (or at best, to have no effect in ameliorating withdrawal symptoms). One would certainly not be motivated by the unsubstantiated speculation in Adams et al. to attempt treatment of drug dependence with an mGluR2/3 antagonist. To the contrary, the consensus view of the leading scientists in the relevant art (as evidenced by the above-noted publications) would undoubtedly have led a skilled artisan away from such a treatment.

The prior art taught away from combining mGluR2/3 antagonist and mGluR5 antagonist

The claimed invention relates to co-administration of an mGluR2/3 antagonist and an mGluR5 antagonist to subjects already suffering from drug dependence. Applicants have previously clarified that Fundytus et al. (British J. Pharmacol. 120:1015-20, 1997) disclosed that simultaneous and chronic administration of morphine and non-selective mGluR antagonist MCPG to normal rats prevented the development of morphine dependence in the rats (as shown in Fig. 1 of Fundytus et al.). In contrast, acute administration of the mGluR antagonist to rats that had already developed morphine dependence produced no therapeutic effect (as shown in Fig. 2 of Fundytus et al.). Applicants further pointed out that the only data in Fundytus et al. that might be relevant to the subject invention are the data shown in Figure 2. Since Figure 2 of Fundytus et al. clearly indicated that administration to drug dependent rats an

mGluR2/3 and mGluR5 dual antagonist has no effect, it is reasonable to conclude that Fundytus et al. taught away from the subject invention.

In the instant office action, the Examiner dismissed Applicants' arguments on the ground that Figure 1 of Fundytus et al. showed that MCPG "can attenuate withdrawal symptoms." Applicants do not dispute that Fundytus et al. reported that normal naïve rats co-administered morphine and MCPG (before the development of morphine dependence) showed less withdrawal symptoms as compared to control rats receiving morphine alone, suggesting that MCPG prevented the development of dependence when co-administered with morphine. However, the preventive effects in normal subjects evidenced by Figure 1 of Fundytus et al. are simply irrelevant to the subject invention which relates to obtaining therapeutic effect in drug dependent subjects (i.e., who were exposed to the drug of abuse and thus developed dependence before the therapeutic intervention). To be abundantly clear, Figure 1 of Fundytus et al. is concerned with normal rats (i.e., rats with no drug dependence). **In addition, the normal rats in Figure 1 of Fundytus et al. were administered MCPG together with morphine (to assess development of morphine dependence).** By contrast, the subject invention is not intended to prevent the development of drug dependence in normal subjects. Instead, the invention is directed to treating subjects who are already drug dependent. **With a purpose of treating drug dependence in addictive subjects as presently claimed, the subjects certainly do not receive their medication together with the very drug on which they are already dependent (e.g., nicotine, cocaine or morphine).** Rather, the drug dependent subjects are administered only the medication (i.e., the combination of an mGluR2/3 antagonist and an mGluR5 antagonist). The presently introduced amendments to the claims should eliminate any doubt or confusion that might otherwise remain regarding such basic difference between the disclosure of Figure 1 in Fundytus et al. and the subject invention.

To summarize, Fundytus et al. taught that MCPG PREVENTED the development of dependence (if co-administered TOGETHER with morphine) and the expression of the withdrawal signs in normal subjects (Fig. 1). However, once dependence has already developed, the drug did NOT TREAT the withdrawal symptoms in the drug dependent subjects (Fig. 2). **In other words, the data in Fundytus et al. that were relied on by the Examiner (i.e., Figure 1) are irrelevant to the subject invention. On the other hand, disclosures in Fundytus et al. that might be relevant to the subject invention (i.e., Figure 2) showed negative results, i.e., teaching away from the claimed invention**.

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From the clarifications presented herein, it is clear that, in spite of the unsupported and unsubstantiated assertion in Adams et al., the prior art actually taught away from treating drug dependence via antagonism of mGluR2/3 receptors. In addition, the prior art certainly did not suggest treating drug dependence by combining mGluR2/3 antagonism and mGluR5 antagonism. In contrast, the relevant data from Fundytus et al. cited by the Examiner unequivocally suggested that such a combination would likely be ineffective.

For all the remarks set forth above and also reasoning submitted in Applicants' previous responses, it is indisputable that the presently claimed invention is by no means obvious over the prior art. Applicants accordingly submit that the presently claimed invention is non-obvious and respectfully request that the rejection be withdrawn.

CONCLUSION

In view of the foregoing, Applicants respectfully submit that the claims now pending in the subject patent application are in condition for allowance, and notification to that effect is earnestly requested. If a telephone conference would expedite

prosecution of this application, please telephone the undersigned attorney at 858-784-2937.

The Director is hereby authorized to charge our Deposit Account No. 19-0962 in the event that there are any charges associated with the present Response or any Response in connection with this application.

Respectfully submitted,

Aug. 27, 2009

Date

Hugh Wang, Ph.D., Reg. No. 47,163

THE SCRIPPS RESEARCH INSTITUTE
10550 North Torrey Pines Road
Mail Drop TPC 8
La Jolla, California 92037
(858) 784-2937

Enclosures

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Attenuation of precipitated morphine withdrawal symptoms by acute i.c.v. administration of a group II mGluR agonist

*†Marian E. Fundytus & *†‡'Terence J. Coderre

*Pain Mechanisms Laboratory, Clinical Research Institute of Montreal, †Department of Psychology, McGill University and ‡Centre de recherche en sciences neurologiques et département de médecine, Université de Montréal, Canada

1 We previously showed that chronic i.c.v. antagonism of metabotropic glutamate receptors (mGluRs) concurrently with s.c. morphine significantly attenuated precipitated withdrawal symptoms. Conversely, acute i.c.v. injection of a selective group II mGluR antagonist just before the precipitation of withdrawal exacerbated abstinence symptoms.

2 In the present study, we showed that acute i.c.v. administration of the non-selective mGluR agonist 1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD), as well as the group II selective agonist (2S,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV), significantly attenuated the severity of precipitated withdrawal symptoms.

3 From these results we hypothesize that chronic opioid treatment may indirectly induce a desensitization of group II mGluRs, which contributes to the development of dependence.

Keywords: Morphine; dependence; (1S,3R)-ACPD; (1S,3S)-ACPD; DHPG; DCG-IV; L-AP4; metabotropic glutamate receptor; precipitated withdrawal

Introduction

Recently, we have shown that chronic i.c.v. antagonism of metabotropic glutamate receptors (mGluRs) concurrently with morphine treatment attenuates the severity of the precipitated withdrawal syndrome (Fundytus & Coderre, 1994; Fundytus *et al.*, 1997). These results suggest that chronic opioid administration may elicit changes in glutamatergic, as well as opioidergic, neurones. Activation of μ-opioid receptors and mGluRs both affect adenosine 3':5'-cyclic monophosphate (cyclic AMP) production and phosphoinositide (PI) hydrolysis. Acute activation of μ-opioid receptors decreases PI hydrolysis (Barg *et al.*, 1992; 1993; 1994), while activation of group I mGluRs stimulates PI hydrolysis (Schoepp & Conn, 1993; Hayashi *et al.*, 1994). Activation of μ-opioid receptors or group II (mGluR2 and 3) or group III (mGluR4, 6, 7 and 8) mGluRs decreases cyclic AMP production (Childers, 1991; Hayashi *et al.*, 1994).

Both opioid receptors and mGluRs are directly coupled to these intracellular second messengers via guanine nucleotide (G) proteins, and opioid receptors and mGluRs are similarly distributed in the brain (Mansour *et al.*, 1995; Masu *et al.*, 1995), suggesting that they may be co-localized within the same cells. Thus, opioid receptors and mGluRs may share common pools of intracellular second messengers, and activity at one type of receptor may modulate activity at the other receptors via actions on second messengers. Whereas acute administration of μ-opioids decreases cyclic AMP production and PI hydrolysis, during chronic administration activity in both systems returns to near control levels (Dixon *et al.*, 1990; Childers, 1991; Barg *et al.*, 1992; 1993; 1994), suggesting that compensatory mechanisms are elicited. Given the similarities of the intracellular events triggered by opioid and mGluRs, and given that chronic antagonism of mGluRs reduces morphine withdrawal symptoms, we hypothesize that, via actions on second messengers, chronic opioid administration may induce a change in the sensitivity of mGluRs, which contributes to the development of opioid dependence.

In the present study, we examined the possibility that desensitization of mGluRs contributes to morphine dependence by assessing the ability of acute i.c.v. treatment, just before the precipitation of withdrawal, with both non-selective and subtype-selective mGluR agonists, to inhibit naloxone-precipitated morphine withdrawal. It is expected that if mGluR desensitization plays a role in opioid dependence, then acute administration of mGluR agonists just before the precipitation of withdrawal should reduce the severity of abstinence symptoms.

In the present study we examined the effect of 1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R)-ACPD), which is a non-selective mGluR agonist that is ten times more potent as an agonist at mGluRs than ionotropic glutamate receptors (Palmer *et al.*, 1989; Schoepp *et al.*, 1991a, b; 1992; Watkins & Collingridge, 1994) on precipitated withdrawal symptoms in chronic morphine-treated rats. The effects of (1S,3R)-ACPD were then compared to a series of subtype-selective mGluR agonists. Group I mGluRs were selectively activated by (RS)-dihydroxyphenylglycine (DHPG) (Schoepp *et al.*, 1994; Watkins & Collingridge, 1994). Group II mGluRs were selectively activated by (1S,3S)-ACPD and (2S,1'R,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV) (Ishida *et al.*, 1993; Watkins & Collingridge, 1994; Pin & Duvoisin, 1995). To activate group III mGluRs, we used L-2-amino-4-phosphonobutyrate (L-AP4) (Nakanishi, 1992; Nakajima *et al.*, 1993; Okamoto *et al.*, 1994; Watkins & Collingridge, 1994).

In these studies, we found that acute administration of either the non-selective agonist (1S,3R)-ACPD, or the mGluR2/3 selective agonist DCG-IV, significantly reduced the severity of the precipitated morphine withdrawal syndrome.

Methods

Subjects and surgery

Subjects were male Long Evans rats (Charles River, PQ), housed 2-4 per cage, maintained on a 12:12 h light:dark cycle (lights on at 06h 00min), with food and water available *ad libitum*. Rats weighed 280-350 g at the time of surgery.

On Day 0, each rat was anaesthetized with sodium pentobarbitone (Somnotol, MTC Pharmaceuticals, 60 mg kg^{-1}), and a 23 gauge stainless steel guide cannula was implanted above the lateral ventricle (AP = -1.3 mm and L = -1.8 mm

[1]Author for correspondence at: Pain Mechanisms Laboratory, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada H2W 1R7.

from bregma, and V = −3.0 mm from the top of the skull; Paxinos & Watson, 1986) so that the 30 gauge injection cannula extended 1 mm below this into the lateral ventricle. While the rat was still under pentobarbitone anaesthesia, one unprimed (i.e. not yet pumping) Alzet osmotic pump containing 50 mg ml^{-1} morphine sulphate was implanted subcutaneously (s.c.) on the back. On Day 1, rats were briefly anaesthetized with halothane and a second Alzet osmotic pump containing 70 mg ml^{-1} morphine sulphate was implanted s.c. on the back of each rat. This two day pump implantation procedure was used to prevent mortality from a lethal concentration of morphine before any tolerance had developed.

Drugs

Morphine sulphate (gift from Sabex, Quebec) was continuously infused subcutaneously (s.c.) at a rate of 10 $\mu l\ h^{-1}$ for a total dose of 36.65 $\mu mol\ day^{-1}$. The mGluR agonists (1S,3R)-ACPD, (1S,3S)-ACPD, DHPG, L-AP4 and DCG-IV were all obtained from Tocris Cookson (Bristol, U.K.). (1S,3R)-ACPD ($n=18$), (1S,3S)-ACPD ($n=18$), DHPG ($n=16$) and L-AP4 ($n=17$) were given intracerebroventricularly (i.c.v.) as an acute injection in a volume of 4 μl at a dose of either 0 (vehicle) ($n=15$), 0.12, 0.6 or 3 nmol, while DCG-IV ($n=11$) was given i.c.v. in a dose of either 4.8 or 24 pmol, since higher doses would produce a non-selective activation of NMDA receptors (Ishida *et al.*, 1993).

Withdrawal measurement

The severity of abstinence symptoms was assessed on the seventh day of morphine treatment following an s.c. injection of 1 mg kg^{-1} naloxone. Behaviour was observed for 10 min before i.c.v. injection of either vehicle or one of the mGluR agonists, 10 min after i.c.v. injection but before naloxone, and for 40 min after the injection of naloxone. Teeth chattering and writhing were timed and combined into a time spent in withdrawal score. The severity of diarrhoea was assessed by the amount of weight lost during the test session. Severity of eye twitch and salivation were rated on a 4 point scale where 0 = absent and 3 = severe. To assess the effects of mGluR agonists on general behaviour, time spent in withdrawal and non-withdrawal (resting, ambulating, rearing, grooming) behaviours were compared between dependent (given morphine) and non-dependent rats given either i.c.v. vehicle or the highest dose of one of the agonists. Testing was performed throughout the 10 min period before the i.c.v. injection, the 10 min after i.c.v. but before naloxone injection, and the 10 min after naloxone injection.

Statistical analysis

Time spent in withdrawal and weight loss were analysed by a 1-way ANOVA performed on each mGluR agonist group with dose as the factor. Significant results were further analysed by *post-hoc* LSD t tests. Time spent in withdrawal and non-withdrawal behaviours were analysed with a mixed ANOVA with morphine treatment and i.c.v. treatment as between subject factors, and time block as a repeated measures factor, again followed by *post-hoc* LSD t tests on significant results. Severity of eye twitch and salivation were analysed with Kruskal-Wallis ANOVA for non-parametric data, followed by Mann-Whitney U-tests on significant results.

Results

Morphine sulphate 36.65 $\mu mol\ day^{-1}$ produced an intense and reliable withdrawal syndrome as indicated by the presence of abstinence symptoms after naloxone injection. As shown in Figure 1a, the non-selective mGluR agonist (1S,3R)-ACPD ($F_{(3,29)}=4.33$, $P<0.05$) and the mGluR2/3 selective agonist DCG-IV ($F_{(2,23)}=26.84$, $P<0.01$) significantly decreased the amount of time spent in withdrawal, with DCG-IV producing the greatest decrease even at the very low doses used. Although DHPG ($F_{(3,27)}=1.67$, $P>0.05$) and L-AP4 ($F_{(3,28)}=1.92$, $P>0.05$) appeared to decrease time spent in withdrawal at the highest dose used, the results failed to reach statistical significance. The severity of diarrhoea was attenuated in DCG-IV-treated rats, as indicated by a reduction in weight loss ($F_{(2,23)}=11.45$, $P<0.01$) in Figure 1b. Figure 2a shows the severity of eye twitch, and Figure 2b shows the severity of salivation. DCG-IV decreased the severity of both eye twitch ($H_{(2,26)}=11.03$, $P<0.01$) and salivation ($H_{(2,26)}=10.61$, $P<0.01$), while the non-selective agonist (1S,3R)-ACPD decreased the severity of eye twitch ($H_{(3,33)}=8.69$, $P<0.05$). In contrast, the mGluR4 selective agonist L-AP4 significantly increased the severity of eye twitch ($H_{(3,32)}=11.69$, $P<0.05$).

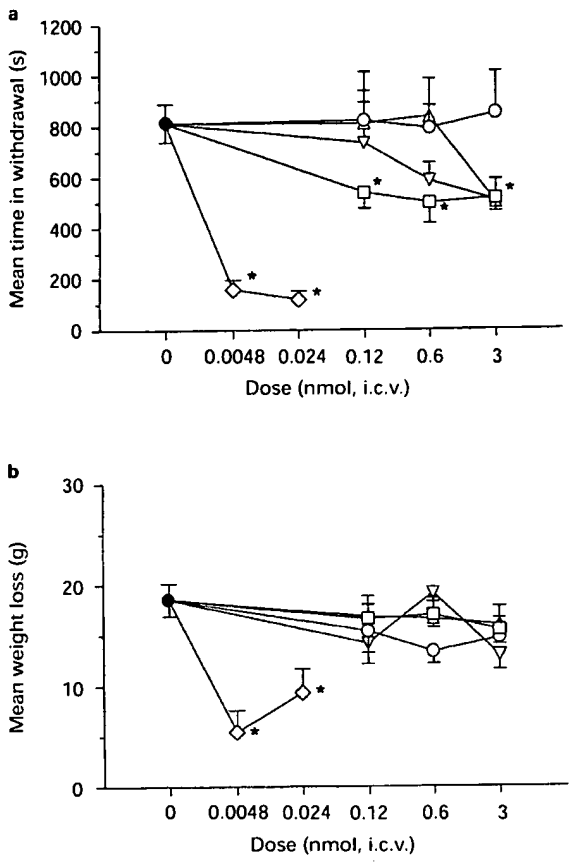

Figure 1 (a) Mean time spent in withdrawal (teeth chattering and writhing combined) during the 40 min withdrawal period in morphine-dependent rats given an acute i.c.v. injection of either vehicle (●; $n=15$), or 0.12, 0.6 or 3 nmol of (1S,3R)-ACPD (□; $n=18$), (1S,3S)-ACPD (○; $n=18$), DHPG (△; $n=16$) or L-AP4 (▽; $n=17$), or 4.8 or 24 pmol of DCG-IV (◇; $n=11$). (b) Mean weight loss during the 40 min withdrawal period in morphine-dependent rats given an acute i.c.v. injection of either vehicle (●), or 0.12, 0.6 or 3 nmol of (1S,3R)-ACPD (□), (1S,3S)-ACPD (○), DHPG (△) or L-AP4 (▽), or 4.8 or 24 pmol of DCG-IV (◇). *Significantly less than control ($P<0.05$, LSD t test) in (a) and (b). Vertical lines show s.e.mean.

To assess the effects of acute i.c.v. injection of mGluR agonists on general behaviour, non-withdrawal and withdrawal behaviours were compared in non-dependent and morphine-dependent rats during the 10 min period before the i.c.v. injection, the 10 min after i.c.v. injection but before naloxone injection, and the 10 min following naloxone injection. Generally, rats were more active earlier in the test session, and by the third time block, after the injection of naloxone, non-

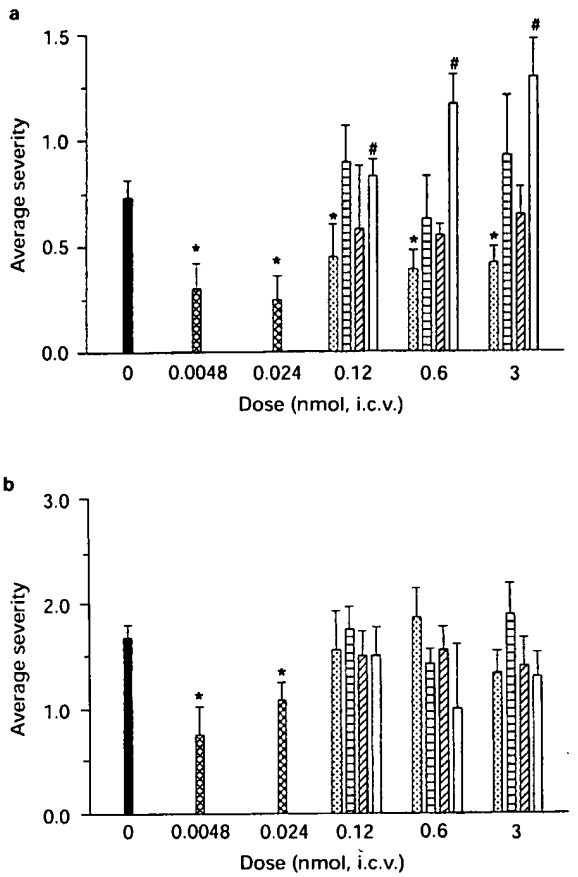

Figure 2 Average severity of (a) eye twitch and (b) salivation during the 40 min withdrawal period in morphine-dependent rats given an acute i.c.v. injection of either vehicle (solid columns), (1S,3R)-ACPD (stippled columns), (1S,3S)-ACPD (horizontally-hatched columns), DHPG (diagonally-hatched columns), L-AP4 (open columns) or DCG-IV (cross-hatched columns). *Significantly less than control ($P<0.05$, LSD t test); #significantly greater than control ($P<0.05$, LSD t test). Each column shows mean ± s.c.mean.

dependent rats spent most of their time resting. Morphine-dependent rats behaved very similarly to non-dependent rats until after the injection of naloxone, at which time they spent more time in withdrawal ($P<0.05$, LSD t test). There were no effects of i.c.v. treatment except that (1S,3R)-ACPD- and DCG-IV-treated rats spent significantly less time in withdrawal than vehicle-treated rats ($P<0.05$, LSD t test; data not shown).

Discussion

The present study shows that the severity of the precipitated morphine withdrawal syndrome was significantly decreased by acute i.c.v. administration of the non-selective mGluR agonist (1S,3R)-ACPD. Although the mGluR1/5 agonist DHPG and the mGluR4 agonist L-AP4 appeared to decrease withdrawal at the highest doses used, the results failed to reach statistical significance. In contrast, the selective mGluR2/3 agonist DCG-IV almost completely eliminated teeth chattering and writhing, as well as significantly attenuating the severity of diarrhoea (indicated by weight loss), despite the fact that DCG-IV, as well as the other agonists, produced negligible effects on non-withdrawal behaviours. The effect of i.c.v. administration of DCG-IV on diarrhoea was surprising since it is generally believed that opioids slow gastro-intestinal motility via local gut actions, with some spinal input. A decrease in withdrawal-induced diarrhoea is consistent with a decreased vagal output, perhaps via the actions of DCG-IV in peri-ventricular regions such as the thalamus and hypothalamus. Although DCG-IV effectively attenuated the severity of withdrawal, the mGluR2/3 agonist (1S,3S)-ACPD failed to do so. This may be due to the fact that (1S,3S)-ACPD is less selective, and stimulates PI hydrolysis and cyclic AMP production via actions at group I mGluRs, as well as decreasing cyclic AMP production via group II mGluRs (Schoepp & Conn, 1993).

Although there is some evidence that DCG-IV is an agonist at NMDA receptors at concentrations above 10 μM *in vitro* (Ishida *et al.*, 1993), our highest dose was only 0.006 μM. Therefore, the doses we used were most likely selective for mGluR2/3 receptors. Thus, the efficacy of DCG-IV can be attributed to its agonist action at mGluR2/3. Moreover, we previously found that while chronic i.c.v. administration of a selective mGluR2/3 antagonist significantly decreased the severity of precipitated withdrawal, acute i.c.v. administration of this antagonist just before the precipitation of withdrawal significantly increased the severity of abstinence symptoms (Fundytus *et al.*, 1997). Taken together, these results support the hypothesis that group II mGluRs may be desensitized during chronic morphine treatment. Chronic antagonism of a receptor is generally believed to induce up-regulation of the receptor. Thus, perhaps chronic antagonism of group II mGluRs induced an up-regulation of these receptors, which counterbalanced the desensitizing effects of morphine treatment, resulting in normally functioning receptors and thus a reduction of withdrawal symptoms. Also, acute administration of DCG-IV in morphine-treated rats may have restored function to group II mGluRs which were no longer sensitive to physiological concentrations of glutamate, enabling these receptors to inhibit cyclic AMP production, and thus decrease the severity of withdrawal.

Group II mGluRs may interact with other systems which have been postulated to be involved in morphine dependence. For example, it has been shown that whereas acute treatment with μ-opioids decreases phosphatidylinositol (PI) hydrolysis (Barg *et al.*, 1992; 1994; Johnson *et al.*, 1994), during chronic opioid treatment PI hydrolysis returns to near control levels (Pelligrini-Giampietro *et al.*, 1988; Narita *et al.*, 1994), and is greatly enhanced during withdrawal (Pelligrino-Giampietro *et al.*, 1988; Narita *et al.*, 1994; Busquets *et al.*, 1995), suggesting that this system and the intracellular messengers produced may play a key role in the development of morphine dependence. Furthermore, we have previously shown that chronic antagonism of group I mGluRs, which are positively coupled to PI hydrolysis (Fundytus & Coderre, 1994), as well as chronic inhibition of protein kinase C (PKC) and intracellular Ca^{2+} (products of PI hydrolysis) (Fundytus & Coderre, 1996), attenuate the development of morphine-dependence. Thus, it is possible that chronic morphine treatment enhances the translocation and activation of protein kinase C (PKC), which may in turn phosphorylate group II mGluRs, leading to desensitization of these receptors.

There is also a large body of evidence suggesting that NMDA receptors are involved in the development of morphine dependence (Trujillo & Akil, 1991; 1994; Fundytus & Coderre, 1994; Mao *et al.*, 1995). It has been suggested that during chronic morphine treatment, an increase in the translocation and activation of PKC induces phosphorylation of the NMDA ion channel, enhancing NMDA receptor activity, and related intracellular messengers such as nitric oxide (NO) (Mao *et al.*, 1995). It has been shown that activation of group II mGluRs inhibits activity at NMDA receptors (Buisson & Choi, 1995). Perhaps a desensitization of group II mGluRs induced by chronic morphine treatment results in a disinhibition of NMDA receptor activity. Thus, acute administration of a group II mGluR agonist just be-

fore the precipitation of withdrawal may not only decrease excitability via a decrease in cyclic AMP production, and thus alleviate abstinence symptoms, but may also serve to decrease excitability by inhibiting NMDA receptor activity.

Our present results provide further support for the hypothesis that mGluR activity may contribute to the development of morphine dependence. Furthermore, our results are of clinical interest. If clinically safe group II mGluR agonists are developed, activation of mGluRs may aid in alleviating withdrawal symptoms of patients and thus make detoxification safer and easier.

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The selective mGlu2/3 receptor agonist LY354740 attenuates morphine-withdrawal-induced activation of locus coeruleus neurons and behavioral signs of morphine withdrawal

Jim Vandergriff, Kurt Rasmussen *

Lilly Research Laboratories, Eli Lilly and Co., Lilly Corporate Center, Indianapolis, IN 426285, USA

Abstract

Naltrexone-precipitated morphine withdrawal induces hyperactivity of locus coeruleus (LC) neurons, as well as a plethora of behavioral withdrawal signs. Previous research has demonstrated that an increased release of glutamate and activation of AMPA receptors, particularly in the LC, play an important role in opiate withdrawal. LY354740 is a novel Group II metabotropic glutamate mGlu2/3 receptor agonist that decreases the release of glutamate. Therefore, we investigated the effect of LY354740 on naltrexone-precipitated morphine-withdrawal-induced activation of LC neurons and behavioral signs of morphine withdrawal. In in vivo recordings from anesthetized rats, pretreatment with LY354740 (3–30 mg/kg, s.c.) dose-dependently attenuated the morphine-withdrawal-induced activation of LC neurons. In unanesthetized, morphine-dependent animals, pretreatment with LY354740 (3–30 mg/kg, s.c.) dose-dependently suppressed the severity and occurrence of many naltrexone-precipitated morphine-withdrawal signs. These results indicate mGlu2/3 receptor agonists: (1) can attenuate the morphine-withdrawal-induced activation of LC neurons and many behavioral signs of morphine withdrawal; and (2) may have therapeutic effects in man for the treatment of opiate withdrawal. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Metabotropic glutamate receptors; Morphine; Withdrawal; Locus coeruleus; Opiates

1. Introduction

The locus coeruleus (LC) is the largest grouping of noradrenergic neurons in the mammalian brain (Dahlstrom and Fuxe, 1965). The cell bodies of the LC are confined to a small area of the pons, but these neurons send extensive projections throughout the neuraxis (Jones and Moore, 1977). These wide ranging projections put the LC in a position to simultaneously influence the activity of a number of brain areas. Hence, the LC has been hypothesized to play a role in a wide variety of behaviors, physiological processes, and brain diseases. In particular, the LC has been shown to play an important role in the expression of opiate withdrawal (Aghajanian, 1978; Rasmussen et al., 1990; Maldonado et al., 1992; Maldonado and Koob, 1993; Rasmussen, 1995). Specifically, the increased release of glutamate and subsequent activation of AMPA receptors is critically involved in opiate withdrawal-induced activation of LC neurons and the behavioral signs of opiate withdrawal (Akaoka and Aston-Jones, 1991; Aghajanian et al., 1994; Rasmussen et al., 1996).

Glutamate receptors have been divided into two broad categories: iontotropic and metabotropic. Iontotropic glutamate receptors contain cation-specific ion channels as a component of their protein complex, while metabotropic glutamate receptors are coupled to G-proteins and modulate intracellular second messenger systems. Thus far, eight different clones for metabotropic glutamate (mGlu) receptors have been isolated (mGlu1-8). Based on agonist interactions, sequence homology, and second messenger coupling, the eight mGlu receptors have been grouped into three large families (Conn and Pin, 1997). Group I mGlu receptors include mGlu1 and mGlu5, Group II mGlu receptors include mGlu2 and mGlu3, and Group III mGlu receptors include mGlu4, 6, 7, and 8. mGlu receptors can differentially modulate synaptic function through both

* Corresponding author. Present address: Lilly Research Centre Limited, Eli Lilly & Co., Erl Wood Manor, Windlesham, Surrey GU20 6PH, UK. Tel.: +44-1276-853540; fax: +44-1276-853525; e-mail: rasmussen_kurt@lilly.com.

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pre- and post-synaptic sites (Schoepp and Conn, 1993; O'Leary et al., 1997). Activation of presynaptic Group II and III mGlu receptors decrease the release of glutamate (Pin and Duvoisin, 1994), while activation of presynaptic Group I mGlu receptors can enhance or depress the release of glutamate (Herrero et al., 1992; Gereau and Conn, 1995). Dube and Marshall (1997) demonstrated that mGlu2/3 receptors inhibit excitatory synaptic transmission in LC neurons, possibly by functioning as autoreceptors for excitatory amino acids. In addition to their presynaptic modulation, activation of postsynaptic mGlu1 and 5 receptors stimulate phosphoinositide hydrolysis, while activation of mGlu2, 3, 4, 6, 7 and 8 receptors inhibit cAMP production (Nakanishi, 1992; Schoepp and Conn, 1993). Therefore, mGlu2/3 receptors can influence a variety of glutamatergic dependent processes by either suppressing postsynaptic neuronal activity or inhibiting presynaptic release of glutamate (Nakanishi, 1992; Pin and Duvoisin, 1994).

Recently, it has been shown that i.c.v. administration of the mGlu2/3 receptor agonist DCG-IV and the non-selective mGlu agonist ACPD attenuate some opiate withdrawal signs in rats (Fundytus and Coderre, 1997). However, a limited number of withdrawal symptoms were examined in this study and ACPD and DCG-IV are not systemically active. Further, DCG-IV is not only an mGlu2/3 agonist, but it is also an agonist for mGlu8 and NMDA receptors, as well as an mGlu7 receptor antagonist (Uyama et al., 1997; Breakwell et al., 1997). Thus, the role of mGlu2/3 receptors in morphine withdrawal and the potential therapeutic uses of mGlu receptor ligands in opiate withdrawal has not been fully explored.

LY354740 is a recently discovered selective mGlu2/3 receptor agonist, which shows no significant iontotropic (iGluR4 and iGluR6) nor mGlu1 and 5 or mGlu4, 6, 7 and 8 receptor activities (Monn et al., 1997; Schoepp et al., 1997). LY354740 is systemically active and highly efficacious in animal models of anxiety and nicotine withdrawal (Helton et al., 1997; Helton et al., 1998). In addition, LY354740 has been shown to decrease the evoked release of glutamate in the striatum (Battaglia et al., 1997). Since increased release of glutamate has been shown to play a critical role in the opiate withdrawal, we examined the effect of LY354740, and its inactive enantiomer LY317207, on the opiate withdrawal-induced activation of LC neurons and behavioral signs of opiate withdrawal.

2. Methods

2.1. Opiate dependence and withdrawal

Opiate dependence was induced in male Sprague–Dawley rats (Charles River, 250–350 g) by the subcutaneous pellet implantation method (Way et al., 1969; Blasig et al., 1973). While under halothane anesthesia, animals were implanted with either morphine pellets (NIDA; 75 mg morphine base, 68.5 mg microcrystalline cellulose, 1.5 mg magnesium stearate, 2.5 mg colloidal silicon dioxide) or placebo pellets (NIDA; 150 mg Avicel PH-102, 1.5 mg magnesium stearate, 0.75 mg colloidal silicon dioxide, 1.75 mg purified water). Two pellets were implanted daily for 2 days. Withdrawal was induced 48 h after the last set of pellets was implanted. To insure steady levels of withdrawal throughout the test period, all four pellets were removed one to 2 h before precipitating withdrawal. Withdrawal was induced by administering the opiate antagonist naltrexone HCl (10 mg/kg; Research Biochemical) subcutaneously.

2.2. Behavioral ratings

For the behavioral assessment of opiate withdrawal, animals were studied in clear plexiglass cages (18 × 10 × 8 in) and remained in these cages for the entire study. Animals were adapted to the cage for 15 min, and were then administered a pretreatment of either LY354740, LY317207, or saline (1 ml/kg, s.c.). Naltrexone was administered 15 min after the pretreatment. Twelve behaviors characteristic of the rat opiate abstinence syndrome were assessed (Himmelsbach et al., 1935; Way et al., 1969; Wei, 1973; Blasig et al., 1973; Aceto et al., 1986). The absolute frequency of eight episodic behaviors was recorded and a score was calculated based on multiples of five incidents (0 = no incidents; 1 = 1–5 incidents; 2 = 6–10 incidents; 3 = 11–15 incidents, etc.). Behaviors scored in this manner were teeth chatter (separated by at least 2 s), jumping, wet-dog shakes, writhing, stereotyped head movements, digging, and erections. Chewing (without any matter in the mouth) was similarly scored in multiples of 100 occurrences. Five withdrawal behaviors could not be defined in discrete episodes and the severity of these behaviors was assessed using a four point scale; 0 = absent; 1 = mild; 2 = moderate; 3 = marked. Behaviors rated in this fashion were lacrimation, ptosis, irritability, diarrhea, and salivation. The amount of weight loss was measured at the end of the rating period (i.e. 1 h after the administration of naltrexone) and a score was calculated based on multiples of 5 g (0 = no loss; 1 = 1–5 g; 2 = 6–10 g; 3 = 11–15 g, etc.).

2.3. In vivo electrophysiological recordings

The animals were anesthetized with chloral hydrate (400 mg/kg, i.p.); supplemental doses of anesthetic were administered through a lateral tail vein as needed. Body temperature was maintained at 35–37°C by a heating pad. Rats were mounted in a stereotaxic apparatus and a cisternal drainage was performed to help prevent tissue swelling. A burr hole was made 1.2 mm posterior

to lambda and 1.1 mm lateral to the midline. The recording electrodes were single-barrel glass micropipettes, broken back to a tip diameter of 2–3 μm and filled with a 2 M NaCl solution. Extracellular single-unit recordings were made in the LC. LC cells were encountered 5.5–6.0 mm below the dural surface (at the above coordinates), just ventral to a zone of relative electrical silence (corresponding to the IVth ventricle), and just medial to cells of the mesencephalic nucleus of the Vth nerve (which could be activated by depression of the mandible). LC cells were identified by their long duration action potential (> 2 ms), characteristic positive-negative waveform, slow and somewhat regular firing pattern, slow firing rate (0.5–5 Hz), and short-latency burst of excitation followed by an extended (1–2 s) quiescent period following compression of the contralateral hindpaw. Recordings of stable, spontaneous firing rates were obtained from each neuron for at least 3 min. The spontaneous rate of different cells was sampled throughout the LC for 15 min before and after the administration of LY354740 or LY317207 and for 60 min after the administration of naltrexone.

2.4. Statistical analysis

Electrophysiological results were analyzed with a two-way ANOVA coupled with Student's post-hoc *t*-test. Composite withdrawal score results were analyzed with a repeated measures MANOVA coupled with Student's post-hoc *t*-test. Individual behavior scores were analyzed with one-way ANOVAs.

3. Results

3.1. Electrophysiological recordings

Pretreatment with LY354740, but not LY317207, significantly attenuated the morphine-withdrawal-induced activation of LC neurons (ANOVA; $F = 7.45$, $P < 0.0001$; Fig. 1). The basal firing rates of LC cells in animals implanted with morphine pellets were significantly lower than in animals receiving placebo pellets ($P < 0.05$), indicating an incomplete development of tolerance to morphine at the time of testing (Fig. 1; baseline). Pretreatment with LY354740 did not alter the firing rates of LC neurons in either morphine or placebo implanted animals (Fig. 1; 0 min post naltrexone). After naltrexone administration, the firing rates of LC cells were significantly elevated for both saline ($P < 0.05$) and LY317207 ($P < 0.05$) pretreated, morphine-dependent animals at 15, 30, 45, and 60 min post naltrexone, but not in placebo-implanted animals (Fig. 1). LY317207 and saline pretreated morphine-dependent animals showed no significant differences in LC firing rates at all time points. Following naltrexone administration, pretreatment with 3 mg/kg LY354740 significantly ($P < 0.05$) attenuated the morphine-withdrawal induced activation of LC firing rates at 15, 30, and 60 min. Pretreatment with 10 mg/kg and 30 mg/kg LY354740 significantly ($P < 0.05$) attenuated the morphine-withdrawal induced activation of LC firing rates at 15, 30, 45 and 60 min time intervals. After naltrexone, the LC firing rates of morphine-dependent rats pretreated with 10 and 30 mg/kg LY354740 were not significantly elevated from placebo implanted animals.

3.2. Behavioral ratings

Pretreatment with LY354740, but not LY317207, significantly attenuated morphine withdrawal signs induced by the administration of naltrexone in morphine dependent animals (MANOVA; $F = 6.11$, $P < 0.0001$; Fig. 2). Many individual withdrawal scores which contributed to the total withdrawal scores were significantly reduced in a dose-dependent manner. Digging was significantly ($P < 0.05$) decreased by all doses of LY354740. Pretreatment with 10 and 30 mg/kg LY354740 significantly ($P < 0.05$) suppressed writhes, salivation, diarrhea, and chews. The occurrence of wet-dog shakes and ptsosis were significantly ($P < 0.05$)

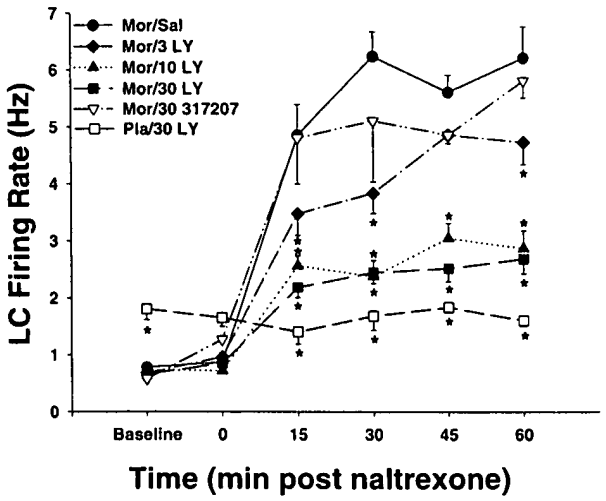

Fig. 1. The activity of locus coeruleus (LC) neurons during naltrexone-precipitated withdrawal in morphine (Mor)-dependent, anesthetized rats pretreated with saline (Sal) (Mor/Sal; ●), 3 mg/kg LY354740 (LY) (Mor/3 LY; ◆), 10 mg/kg LY (Mor/10 LY; ▲), 30 mg/kg LY (Mor/30 LY; ■), or 30 mg/kg 317207 mg/kg (Mor/30 317207; △). The activity of LC neurons in animals receiving placebo (Pla) pellets and treatment with 30 mg/kg LY followed by naltrexone is also shown (Pla/30 LY; □). Values are expressed as mean + SE (some error bars omitted for clarity; $n = 4$ to 36 neurons at each time point). Significant differences ($P < 0.05$) are denoted by an asterisk (*).

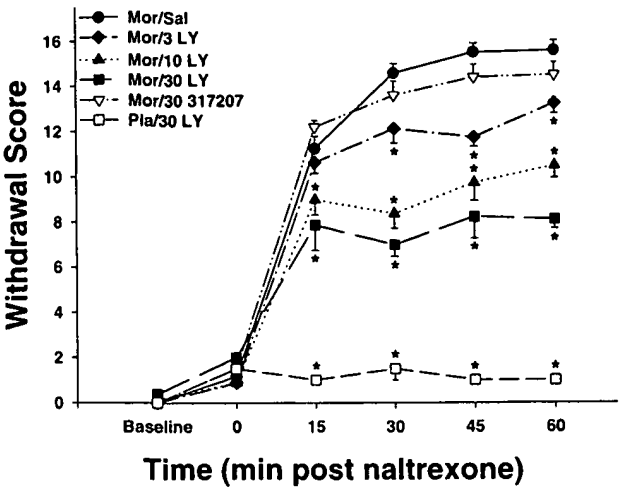

Fig. 2. Effects of pretreatment with saline (Mor/Sal; ●; $n = 8$), 3 mg/kg LY (Mor/3 LY; ◆; $n = 8$), 10 mg/kg LY (Mor/10 LY; ▲; $n = 8$), 30 mg/kg LY (Mor/30 LY;■; $n = 8$), or 30 mg/kg 317207 mg/kg (Mor/30 317207; △; $n = 8$) on naltrexone-precipitated withdrawal signs in morphine-dependent rats. Values are expressed as mean ± SE (some error bars omitted for clarity). Significant differences ($P < 0.05$) are denoted by an asterisk (*).

suppressed by pretreatment with 30 mg/kg LY354740. Chatters, jumps, lacrimination, irritability, erections, and weight loss were not significantly reduced by any dose of LY354740 (Table 1). No individual withdrawal behaviors were significantly reduced by pretreatment with LY317207. Doses of LY354740 up to 100 mg/kg had no significant effect on locomotor activity or the righting reflex and produced no signs of sedation (data not shown).

4. Discussion

The results of this study demonstrate that activation of mGlu2/3 receptors can have a strong influence on opiate withdrawal. In morphine dependent rats, LY354740, but not its inactive isomer LY317207, significantly reduced naltrexone-precipitated activation of LC neurons. The typical 5 to 6-fold increase in LC neuronal firing rates seen during morphine withdrawal was dose-dependently suppressed by acute pretreatment with LY354740. This finding is consistent with previous research demonstrating that presynaptic mGlu receptors can act as glutamate autoreceptors to inhibit activation of LC neurons (Dube and Marshall, 1997). Since there is an increase release of glutamate in the LC during morphine withdrawal (Aghajanian et al., 1994), and LY354740 can reduce the veratridine-stimulated release of glutamate in vivo (Battaglia et al., 1997), it seems likely that LY354740 attenuates the morphine withdrawal induced activation of LC neurons, at least in part, by decreasing the release of glutamate. However, presynaptic Group II mGlu receptors have also been shown to reduce transmission at inhibitory GABA synapses (Salt and Eaton, 1995). Since the LC receives GABA containing afferents (Ennis and Aston-Jones, 1989), LY354740 may also affect the release of GABA in the LC. In addition, indirect effects of presynaptic mGlu receptors on the release of other neurotransmitters in the LC may also play a role in the effects of LY354740. Further studies are needed to determine if LY354740 affects the release of glutamate, GABA, and/or other neurotransmitters in the LC during opiate withdrawal.

LY354740, but not LY317207, dose-dependently inhibited the overall withdrawal score, as well as several individual withdrawal behaviors seen during naltrexone-precipitated morphine withdrawal. The maximal decrease in the overall withdrawal score produced by LY354740 is comparable to the maximal suppression of opiate withdrawal achieved with NMDA and AMPA antagonists (Rasmussen et al., 1996). However, unlike NMDA and AMPA antagonists, even very high doses of LY354740 did not produce sedation or ataxia. In addition to reducing total withdrawal scores, LY354740 significantly suppressed many of the individual withdrawal symptoms. Pretreatment with 10 mg/kg, i.p., decreased writhes, digging, salivation, diarrhea and chews. At the highest dose tested (30 mg/kg, i.p.), wet dog shakes and ptsosis were also significantly reduced. Thus, LY354740 suppressed many of the same withdrawal behaviors as NMDA and AMPA antagonists (Rasmussen et al., 1991; Rasmussen et al., 1996).

While the presynaptic actions of LY354740 in the LC are likely to play a major role in its suppression of morphine withdrawal symptoms, the activation of postsynaptic mGlu2/3 receptors in the LC may also be involved. Recent evidence has shown that mGlu2/3 agonists, including LY354740, inhibit cAMP formation and adenylate cyclase (AC) activity via postsynaptic receptors (Schaffhauser et al., 1997). Upregulation of cAMP and AC pathways in the LC play an important role in the development and expression of morphine dependence (Nestler, 1996; Nestler and Aghajanian, 1997). For example, chronic morphine administration increases levels of AC and cAMP-dependent protein kinases activity in the LC and intra-LC administration of cAMP-dependent protein kinase inhibitors attenuates opiate withdrawal (Lane-Ladd et al., 1997; Punch et al., 1997). Therefore, LY354740 may attenuate the morphine withdrawal-induced activation of LC neurons by not only inhibiting the release of glutamate, but also by reducing the production of cAMP in LC neurons.

In addition to effects in the LC, actions of LY354740 in other brain areas may play a role in its suppression of morphine withdrawal behaviors. Other potential sites of action of LY354740 include those with the highest densities of mGlu2/3 receptors, including the cerebral cortex, olfactory areas, hippocampus, substantia nigra,

Table 1
Effect of pretreatment with saline, LY354740, or LY317207 on individual withdrawal signs[a]

Behaviors	Saline		3 mg/kg		10 mg/kg		30 mg/kg		317207	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Chatter	4.42	1.39	5.86	1.60	5.88	1.37	8.75	3.21	5.70	1.07
Jump	1.00	0.44	1.50	0.53	0.00	0.00	0.13	0.13	1.60	0.52
Writh	6.25	1.30	4.50	1.65	0.88*	0.52	0.25*	0.16	10.50	2.85
Dog shake	11.25	2.36	7.25	1.78	11.38	3.47	4.50*	1.43	9.40	2.36
Ptosis	9.75	0.22	8.75	0.53	8.50	0.53	7.50*	1.00	9.20	0.57
Digging	46.17	10.42	21.63*	8.20	19.00*	7.40	17.50*	4.51	27.20	7.33
Lacrimination	0.83	0.34	1.00	0.38	0.38	0.18	0.50	0.19	0.70	0.52
Salivation	7.42	0.42	7.00	0.65	4.50*	0.71	4.13*	0.30	7.10	0.59
Irritability	5.25	0.60	4.75	0.86	5.25	0.60	3.38	0.65	5.70	0.76
Erection	1.67	0.57	2.00	0.65	1.87	0.44	2.87	1.91	2.30	0.60
Diarrhea	7.25	0.52	6.00	0.57	2.75*	0.25	2.88*	0.61	7.80	0.33
Chews	528.17	80.11	431.50	124.63	260.50*	36.93	247.63*	56.36	540.50	47.57
Weight (g)	21.67	1.83	20.50	1.41	18.75	1.47	18.75	1.62	24.60	1.83

[a] Values represent total expression (mean ± SE) of each behavior for the 60-min period following naltrexone administration.
* Significant differences ($P<0.05$).

habenula, fimbria, interpeduncular nucleus, inferior olive, cerebellum, and spinal cord (Petralia et al., 1995). Importantly, mGlu2/3 receptors are located in some areas thought to be involved in opiate withdrawal behaviors such as the hypothalamus, amygdala, periaquaductal grey area, and spinal cord (Maldonado et al., 1992; Petralia et al., 1995). One potentially important site for the actions of LY354740 during morphine withdrawal is the nucleus paragigantocellularis (PGi). The PGi contains mGlu2/3 receptors, sends a major glutamatergic afferent to the LC, and lesions of the PGi reduce morphine withdrawal symptoms (Ennis and Aston-Jones, 1988; Rasmussen and Aghajanian, 1989; Petralia et al., 1995). Thus, activation of mGlu2/3 receptors in the PGi may reduce subsequent release of glutamate in the LC and attenuate activation of LC neurons. Additional experiments will be needed to address this hypothesis.

The relative contribution of mGlu2 versus mGlu3 receptors to the action of LY354740 can not be determined from this study. However, given that LY354740 has a 6-fold higher affinity for mGlu2 than mGlu3 receptors (Schoepp et al., 1997) and that mGlu2 and 3 receptors have a differential distribution in the brain (Ohishi et al., 1993a,b), activation of mGlu2 and mGlu3 receptors may have different effects on morphine withdrawal. Additional studies with compounds that have greater selectively for mGlu2 or mGlu3 receptors will be needed to elucidate the role of each receptor subtype in opiate withdrawal.

In conclusion, the selective mGlu2/3 agonist, LY354740, but not its inactive isomer LY317207, suppressed the morphine-withdrawal-induced activation of LC neurons and many morphine withdrawal symptoms. These results indicate that mGlu2/3 receptors can have a strong influence on opiate withdrawal and that LY354740, and other mGlu2/3 agonists, may have therapeutic potential for the treatment of opiate withdrawal.

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Group II Metabotropic and α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionate (AMPA)/Kainate Glutamate Receptors Regulate the Deficit in Brain Reward Function Associated with Nicotine Withdrawal in Rats

PAUL J. KENNY, FABRIZIO GASPARINI, and ATHINA MARKOU

Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California (P.J.K., A.M.); and Nervous System Research, Novartis Biomedical Institutes, Basel, Switzerland (F.G.)

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ABSTRACT

This study investigated the role of ionotropic and metabotropic glutamate receptors in the deficits in brain reward function, as measured by elevations in intracranial self-stimulation (ICSS) reward thresholds, associated with nicotine withdrawal. The group II metabotropic glutamate (mGluII) receptor agonist LY314582 [a racemic mixture of LY354740 ([+]-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid)] (2.5–7.5 mg/kg) precipitated withdrawal-like elevations in ICSS thresholds, a sensitive measure of reward function, in nicotine-dependent but not control rats. LY314582 did not affect response latencies, a measure of performance in the ICSS paradigm. Bilateral microinfusion of LY314582 (10–100 ng/side) into the ventral tegmental area likewise precipitated dose-dependent threshold elevations in nicotine-dependent rats. Furthermore, a single injection of the mGluII receptor antagonist LY341495 (2S-2-amino-2-[1S,2S-2-carboxycyclopropan-1-yl]-3-[xanth-9-yl]propionic acid) (1 mg/kg) attenuated the threshold elevations observed in rats undergoing spontaneous nicotine withdrawal. mGluII receptors are primarily located on glutamatergic terminals throughout the mesocorticolimbic system, where they act as inhibitory autoreceptors. To investigate whether mGluII receptors contributed to nicotine withdrawal by decreasing glutamatergic transmission, we next examined whether direct blockade of postsynaptic glutamate receptors precipitated withdrawal-like reward deficits in nicotine-dependent rats. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX; 0.01–1 mg/kg) precipitated withdrawal-like threshold elevations in nicotine-dependent but not control rats, whereas 6-methyl-2-[phenylethynyl]-pyridine (MPEP; 0.01–3 mg/kg) and dizocilpine (MK-801; 0.01–0.2 mg/kg), antagonists at metabotropic glutamate 5 and *N*-methyl-D-aspartate receptors, respectively, did not. Overall, these data demonstrate that mGluII receptors play an important role in the reward deficits associated with nicotine withdrawal. Furthermore, it is likely that mGluII receptors generate this reward deficit, at least in part, by decreasing glutamate transmission at AMPA/kainate receptors.

There is now compelling evidence that the aversive withdrawal syndrome observed during periods of nicotine abstinence contributes to the persistence of the tobacco habit in smokers (Hughes, 1992; Kenny and Markou, 2001). Nicotine withdrawal was shown to precipitate a deficit in brain reward function, as measured by elevations in intracranial self-stimulation (ICSS) reward thresholds, similar to that observed in rats undergoing withdrawal from other major drugs of abuse (Epping-Jordan et al., 1998). Moreover, avoidance and alleviation of this deficit in brain reward function has been proposed as a major motivational factor contributing to craving, relapse, and continued tobacco consumption in human smokers (Epping-Jordan et al., 1998; Kenny and Markou, 2001). In contrast to the intense investigations into the mechanisms by which acute nicotine produces its rewarding effects, little is known concerning the mechanisms mediating the reward deficits associated with nicotine withdrawal.

Most drugs of abuse have been shown to stimulate excitatory glutamatergic transmission throughout brain reward circuitries (Kalivas and Duffy, 1998; Wolf et al., 2000). Increases in glutamatergic transmission have been shown to play an important role in mediating the positive reinforcing actions of addictive drugs (Harris and Aston-Jones, 2003).

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ABBREVIATIONS: ICSS, intracranial self-stimulation; VTA, ventral tegmental area; mGluII, group II metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; mGlu5, metabotropic glutamate 5 receptors; MK-801, dizocilpine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; MPEP, 6-methyl-2-[phenylethynyl]-pyridine; ANOVA, analysis of variance.

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Indeed, it is thought that nicotine elicits its rewarding actions, at least in part, by activating nicotinic acetylcholine receptors located on glutamate terminals in the ventral tegmental area (VTA), thereby potentiating excitatory glutamatergic transmission in this reward-relevant brain site and increasing mesoaccumbal dopamine transmission (Mansvelder and McGehee, 2000). Accordingly, blockade of glutamatergic transmission reduced nicotine's stimulatory action on mesoaccumbens dopamine transmission (Schilstrom et al., 1998) and attenuated the rewarding actions of nicotine and other drugs of abuse (Chiamulera et al., 2001; Laviolette and van der Kooy, 2003; Paterson et al., 2003).

It has been suggested that the neuroadaptations that occur during prolonged exposure to drugs of abuse, which give rise to the deficits in brain reward function associated with withdrawal, may reside in the same neural elements that mediate the acute rewarding actions of these drugs (Koob and Le Moal, 2001). Indeed, in contrast to nicotine's acute stimulatory effects, nicotine withdrawal attenuated mesoaccumbens dopamine transmission (Hildebrand et al., 1997), an action likely to contribute to the reward and motivational deficits associated with nicotine withdrawal (Kenny and Markou, 2001). Therefore, because increases in excitatory glutamatergic transmission are believed to play an important role in the reinforcing actions of acute nicotine, we hypothesized that withdrawal from nicotine is associated with decreased glutamatergic transmission in brain reward circuitries, which contributes to the reward deficits observed during withdrawal. To test this hypothesis, the effects of a group II metabotropic glutamate (mGluII) receptor agonist were examined in nicotine-treated and control rats. mGluII receptors, comprising of mGlu2 and mGlu3 receptors, are inhibitory autoreceptors located on glutamate terminals throughout the mesocorticolimbic system, where they act to decrease excitatory glutamatergic transmission (Bonci et al., 1997; Wigmore and Lacey, 1998). Because mGluII receptor agonists decrease glutamatergic transmission in brain reward circuitries (Manzoni and Williams, 1999), we predicted that activation of these receptors would precipitate ICSS threshold elevations in nicotine-dependent rats similar to those observed in rats during spontaneous nicotine withdrawal, whereas blockade of these receptors would reverse the threshold elevations associated with spontaneous nicotine withdrawal. To further investigate the role of glutamatergic transmission in nicotine withdrawal, we also examined whether direct blockade of glutamatergic transmission at postsynaptic NMDA, AMPA/kainate, and metabotropic glutamate 5 (mGlu5) receptors precipitated withdrawal-like ICSS threshold elevations in nicotine-dependent rats.

Materials and Methods

Animal Housing. Subjects were 149 male Wistar rats weighing 300 to 320 g at the start of each experiment. Rats were obtained from Charles River Laboratories (Raleigh, NC) and were housed in groups of two or three per cage, with food and water available ad libitum. Animals were maintained in a temperature-controlled vivarium under a 12-h light/dark cycle (lights off at 10:00 AM). Animals were tested during the dark portion of the light/dark cycle, except for the spontaneous nicotine withdrawal experiment when rats were tested at time points according to the experimental design. All animals were treated in accordance with the guidelines of the National Institutes of Health regarding the principles of animal care. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care.

Drugs. (−)-Nicotine hydrogen tartrate salt ([−]-1-methyl-2-[3-pyridyl] pyrrolidine) and dizocilpine ([+]-MK-801 hydrogen maleate; [(5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine hydrogen maleate]) were purchased from Sigma-Aldrich (St. Louis, MO); LY341495 (2*S*-2-amino-2-[1*S*,2*S*-2-carboxycyclopropan-1-yl]-3-[xanth-9-yl]propionic acid) and NBQX disodium (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline disodium) were purchased from Tocris Cookson (Ballwin, MO). LY314582 (the racemic mixture of LY354740 [(+)-2-aminobicyclo(3.1.0)hexane-2,6-dicarboxylic acid]) and 6-methyl-2-[phenylethynyl]-pyridine (MPEP) were synthesized by one of the coauthors (F. Gasparini). Drugs were prepared immediately before each administration. For systemic administration, all drugs were dissolved in sterile water and administered by intraperitoneal injection, in a volume of 1 ml/kg body weight, 30 min before the experimental session. For direct intra-VTA administration, LY314582 was dissolved in artificial cerebrospinal fluid of the following composition: 126.6 mM NaCl, 27.4 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO_4, 0.89 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.48 mM Na_2HPO_4 and 7.1 mM glucose, pH 7.4. Rats received intra-VTA injections immediately before the initiation of the experimental session. Unless otherwise stated, drug doses refer to the salt form.

Apparatus. Intracranial self-stimulation training and testing took place in 16 Plexiglas operant chambers (25 × 31 × 24 cm) (MED Associates, St. Albans, VT). The floors of the operant chambers were constructed of parallel aluminum rods spaced 1.25 cm apart. One wall contained a metal wheel manipulandum that required 0.2 N force to rotate it one-quarter of a turn. The wheel (5 cm in width) extended out of the wall ~3 cm. Each testing chamber was enclosed within a light- and sound-attenuated chamber (62 × 63 × 43 cm). Intracranial stimulation was delivered by constant current stimulators (Stimtech model 1200; San Diego Instruments, San Diego, CA). Subjects were connected to the stimulation circuit through flexible bipolar leads (Plastics One, Roanoke, VA) attached to gold-contact swivel commutators (model SL2C; Plastics One) mounted above the chamber. The stimulation parameters, data collection, and all test session functions were controlled by a microcomputer.

Placement of Electrodes and Cannulas. Rats were anesthetized by inhalation of 1 to 3% halothane in oxygen and positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The incisor bar was adjusted to 5 mm above the interaural line, and the skull exposed. Stainless steel bipolar electrodes (11 mm in length) were implanted into the posterior lateral hypothalamus (AP −0.5 mm from bregma; ML ±1.7 mm; DV 8.3 mm from dura), according to the atlas of Pellegrino et al. (1979). For the VTA infusion experiment, bilateral stainless steel guide cannulas (23-gauge, 14 mm in length) were implanted 3 mm above the VTA (AP −3.2 mm from bregma; ML ±1.7 mm; DV 5.3 mm from skull surface; angle of 10° from midline), at the same time that ICSS electrodes were implanted. Four indentations were made in the skull to accommodate screws that together with the application of dental acrylic, held the electrode and cannulas in place. Cannulas were kept patent using 14-mm-long stainless steel stylets (30-gauge). Animals were allowed to recover from surgery for at least 7 days before training in the ICSS paradigm.

Osmotic Mini-Pump Surgery. Rats were anesthetized by inhalation of 1 to 3% halothane in oxygen and prepared with Alzet osmotic mini-pumps [model 2ML4 (28 day); Alza, Palo Alto, CA] placed subcutaneously (back of the animal parallel to the spine). Pumps were filled with either sterile water or nicotine salt solution. The concentration of the nicotine salt solution was adjusted according to animal body weight, resulting in delivery of 9 mg/kg/day (3.16 mg/kg, free base). This dose of nicotine maintains stable plasma levels (~44 ng/ml) comparable with those obtained in human smokers consuming approximately 30 cigarettes per day (Benowitz, 1988). After mini-pump implantation (or removal), the surgical wound was

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closed with 9-mm stainless steel wound clips (BD Biosciences Primary Care Diagnostics, Sparks, MD) and treated with topical antibiotic (Bacitracin) ointment.

ICSS Reward Threshold Procedure. Animals were trained to respond according to a modification of the discrete-trial current-threshold procedure of Kornetsky and Esposito (1979). Briefly, a trial was initiated by the delivery of a noncontingent electrical stimulus. This electrical reinforcer had a train duration of 500 ms and consisted of 0.1-ms rectangular cathodal pulses that were delivered at a frequency of 50 to 100 Hz. The frequency of the stimulation was selected for individual animals so that current-intensity thresholds of each subject were within 85 to 160 μA, and thus allowed both threshold elevations and lowerings to be detected. This frequency was held constant throughout the experiment. A one-quarter turn of the wheel manipulandum within 7.5 s of the delivery of the noncontingent electrical stimulation resulted in the delivery of an electrical stimulus identical in all parameters to the noncontingent stimulus that initiated the trial. After a variable intertrial interval (7.5–12.5 s, average of 10 s), another trial was initiated with the delivery of a noncontingent electrical stimulus. Failure to respond to the noncontingent stimulus within 7.5 s resulted in the onset of the intertrial interval. Responding during the intertrial interval delayed the onset of the next trial by 12.5 s. Current levels were varied in alternating descending and ascending series. A set of three trials was presented for each current intensity. Current intensities were altered in 5-μA steps. In each testing session, four alternating descending and ascending series were presented. The threshold for each series was defined as the midpoint between two consecutive current intensities that yielded "positive scores" (animals responded for at least two of the three trials) and two consecutive current intensities that yielded "negative scores" (animals did not respond for two or more of the three trials). The overall threshold of the session was defined as the mean of the thresholds for the four individual series. Each testing session was ~30 min in duration. The time between the onset of the noncontingent stimulus and a positive response was recorded as the response latency. The response latency for each test session was defined as the mean response latency of all trials during which a positive response occurred. After establishment of stable ICSS reward thresholds, rats were tested in the ICSS procedure once daily except for the spontaneous nicotine withdrawal experiment when rats were tested at time points according to the experimental design.

Intracerebral Injection Procedure. All injections were administered bilaterally in a volume of 0.5 μl/side given over 66 s through 17-mm injectors. The injectors were connected to calibrated polyethylene-10 tubing preloaded with drug solution and protruded 3 mm below the ends of the cannulas into the VTA. After infusion, the injectors were kept in place for an additional 60 s to allow for drug diffusion and to minimize diffusion along the injection tract when pulling out the injector. Injectors were then removed and replaced with 14-mm wire stylets, and the animals were placed directly into the ICSS testing apparatus. Injections were made using a microinfusion pump (model 975; Harvard Apparatus Inc., Holliston, MA).

Systemic Drug Administration Experiments. These experiments investigated whether nicotine withdrawal, as measured by elevations in ICSS thresholds, could be precipitated in nicotine-treated rats by systemic administration of an agonist at mGluII receptors (LY314582), or antagonists at mGlu5 (MPEP), NMDA (dizocilpine), or AMPA/kainate (NBQX) glutamate receptors. For each drug tested, rats were trained in the ICSS paradigm until stable baseline responding was achieved, defined as ≤10% variation in thresholds for three consecutive days and requiring approximately 14 days of daily testing. In each case, drug-naïve rats were then assigned to two separate groups such that there was no difference in mean baseline ICSS thresholds or body weight between groups. One group was then prepared with subcutaneous osmotic mini-pumps delivering vehicle and the second group with mini-pumps delivering 9 mg/kg/day nicotine hydrogen tartrate (3.16 mg/kg/day nicotine free base). There was a minimum 7-day interval after mini-pump implantation, during which ICSS reward thresholds continued to be measured daily, before the effect of any systemically administered drug on reward thresholds was evaluated. This time period was sufficient to produce robust elevations in thresholds in nicotine-treated but not vehicle-treated rats upon abrupt removal of mini-pumps (i.e., spontaneous withdrawal) or administration of nicotinic receptor antagonists (i.e., precipitated withdrawal; Epping-Jordan et al., 1998). Separate groups of nicotine-treated rats and their corresponding nicotine-naive control group were then injected intraperitoneally with the mGluII receptor agonist LY314582 (0, 2.5, 0.5, and 7.5 mg/kg; $n = 9$ nicotine, $n = 11$ control), the mGlu5 receptor antagonist MPEP (0, 0.01, 0.05, and 0.1 mg/kg; $n = 8$ nicotine, $n = 7$ vehicle or 0, 0.5, 1, 2, and 3 mg/kg; $n = 13$ nicotine, $n = 13$ vehicle), the NMDA receptor antagonist dizocilpine (0, 0.01, 0.05, 0.1, 0.175, and 0.2 mg/kg; $n = 10$ nicotine, $n = 9$ control), or the AMPA/kainate receptor antagonist NBQX (0, 0.01, 0.025, 0.05, 0.075, 0.1, 0.5, and 1 mg/kg; $n = 10$ nicotine, $n = 12$ control) according to within-subjects Latin-square designs and ICSS thresholds were evaluated 30 min later. A minimum of 48 h was allowed between each injection in the Latin-square design, during which ICSS thresholds continued to be measured daily, to ensure that ICSS thresholds returned to baseline levels before the next drug administration. The doses of LY314582 and MPEP were chosen based on a previous study demonstrating that ≥10 mg/kg LY314582 and ≥3 mg/kg MPEP elevated ICSS thresholds in drug-naïve rats (Harrison et al., 2002). For the potential demonstration of statistical interaction effects, it was important to include doses of the test drugs that did not alter thresholds under baseline conditions.

Intraventral Tegmental Area Administration Experiment. After stable baseline ICSS responding was achieved (≤10% variation in threshold for three consecutive days), rats ($n = 15$) with bilateral cannulas directed toward the VTA were allocated to two groups such that there were no differences in mean baseline reward thresholds or body weight between groups. One group was then prepared with subcutaneous osmotic mini-pumps delivering vehicle and a second group with mini-pumps delivering nicotine (3.16 mg/kg/day nicotine free-base). Animals again were tested in the ICSS paradigm each day for 7 days before drug treatment. Both groups of rats were then injected directly into the VTA, as described above, with LY314582 (0, 10, 50, and 100 ng/side; $n = 7$ nicotine, $n = 8$ control) according to a within-subjects Latin square design, and ICSS reward thresholds were evaluated immediately postinjection. There was a minimum 48-h interval between each injection, during which ICSS thresholds continued to be measured, to allow thresholds to return to baseline levels before further drug tests. At the conclusion of the experiment, all animals were anesthetized and their brains removed and immediately placed on ice. The brains were cut in 50-μm sections, and placements of the injectors and the electrodes were examined (Fig. 1 for histological verification of injection sites). Only those rats with injection tips located within the VTA were included in statistical analyses.

Spontaneous Nicotine Withdrawal Experiment. Osmotic mini-pumps were surgically removed from nicotine-treated rats ($n = 15$) (defined as rats having been prepared with mini-pumps delivering 3.16 mg/kg/day nicotine free-base for at least 7 days) or corresponding control rats ($n = 17$; rats prepared with vehicle-containing mini-pumps). All rats were then tested in the ICSS procedure at 12, 18, 24, 36, 48, and 72 h after the removal of osmotic mini-pumps. These time points were chosen based on the time course of threshold elevations previously observed during spontaneous nicotine withdrawal after removal of nicotine-delivering osmotic mini-pumps (Harrison et al., 2001). Based on the ICSS reward thresholds obtained at the 12-h time point, nicotine-withdrawing rats were allocated to two groups such that there was no difference in the magnitude of reward threshold elevations between each group (117.67 ± 3.1%, $n = 8$; 119.93 ± 3.5%, $n = 7$). Similarly, control rats were allocated to two groups such that there was no difference in mean reward thresholds between these groups (106.45 ± 5.2%, $n = 7$; 103.63 ± 3.6%, $n = 10$). Thirty min before being tested at the 18-h

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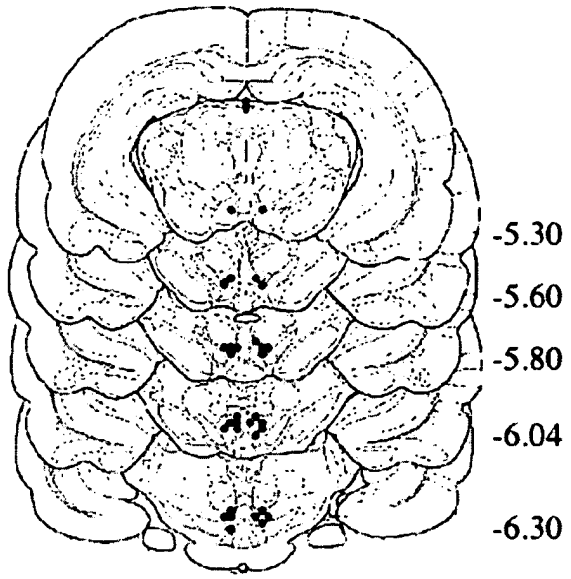

Fig. 1. Diagrammatic representation of coronal sections from the rat brain showing histological reconstruction of the injection sites in the ventral tegmental area. Black circles indicate locations of injector tips located inside the VTA (5.30–6.30 mm posterior to bregma, according to the atlas of Paxinos and Watson, 1986), and included in statistical analysis. Data from rats with injection sites located outside the VTA were removed from the analyses.

time point, one group of nicotine withdrawing and one group of control rats were injected with LY341495 (1 mg/kg); the remaining rats were injected with vehicle.

Statistical Analyses. Mean raw thresholds and response latencies (± S.E.M.) are presented for each experiment in the results section. For all experiments, except the spontaneous nicotine withdrawal experiment, percentage of change from baseline reward threshold was calculated by expressing the drug-influenced raw threshold scores as a percentage of the previous day's threshold (i.e., a drug-free baseline threshold). These percentages of baseline scores were subjected to two-factor repeated-measures analyses of variance (ANOVA), with treatment drug dose as the within-subjects factor and pump content (nicotine or control) as the between-subjects factor. For the spontaneous nicotine withdrawal experiment, percentage change from baseline reward threshold was calculated by expressing the threshold scores obtained at each time point during withdrawal as a percentage of thresholds for each rat on the day immediately before mini-pump removal. These percentages of baseline scores were subjected to three-factor repeated measures ANOVA. The within-subjects factor was the time after mini-pump removal, and the two between-subjects factors were pump content (nicotine or vehicle) and acute drug treatment (LY314582 or vehicle). For all experiments, response latency data were analyzed in the same manner as the threshold data. After statistically significant effects in the ANOVAs, post hoc comparisons among means were conducted with the Fisher's least significant difference test. The level of significance was set at 0.05.

Results

Systemic Administration of the mGluII Receptor Agonist LY314582 Precipitated Elevations in ICSS Thresholds in Nicotine-Treated but Not Control Rats. Mean (±S.E.M.) raw reward thresholds before treatment with the mGluII receptor agonist LY314582 for control and nicotine-treated rats were 98.9 ± 11.6 and 96.3 ± 12.3 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.38 ± 0.2 and 3.21 ± 0.14 μA, respectively. Intraperitoneal administration of LY314582 (2.5–7.5 mg/kg) elevated ICSS reward thresholds in nicotine-treated but not control rats. This effect was reflected in a statistically significant effect of group [$F_{(1,18)} = 7.43, p < 0.05$], a significant effect of dose [$F_{(3,54)} = 5.02, p < 0.005$], and a significant group × dose interaction [$F_{(3,54)} = 2.79, p < 0.05$]. Post hoc analysis revealed that the highest dose of LY314582 (7.5 mg/kg) elevated reward thresholds in nicotine-treated rats compared with vehicle treatment ($p < 0.01$) and compared with control rats tested with the same dose ($p < 0.01$) (Fig. 2). In contrast to its effects on reward thresholds, LY314582 had no effect on response latencies in nicotine-treated or control rats [$F_{(3,54)} = 0.59$, N.S.] at any dose tested (data not shown).

Ventral Tegmental Area Administration of the mGluII Receptor Agonist LY314582 Precipitated Elevations in ICSS Reward Thresholds in Nicotine-Treated but Not Control Rats. Mean (±S.E.M.) raw reward thresholds before intra-VTA administration of LY314582 for control and nicotine-treated rats were 115.3 ± 12.2 and 113.5 ± 19.0 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.55 ± 0.29 and 3.15 ± 0.13 s, respectively. Bilateral microinfusion of LY314582 (10–100 ng/side) directly into the VTA significantly elevated reward thresholds in nicotine-treated but not control rats (Fig. 3). Again, there were significant effects of group [$F_{(1,13)} = 4.81, p < 0.05$], dose [$F_{(3,39)} = 4.77, p < 0.01$], and a significant group × dose interaction [$F_{(3,39)} = 3.82, p < 0.05$]. Post hoc analyses revealed that doses of 50 and 100 ng/side LY314582 were sufficient to elevate reward thresholds in nicotine-treated rats without affecting thresholds in control rats. LY314582 had no effect on response latencies [$F_{(3,39)} = 1.94$, N.S.] in nicotine-treated or control rats after VTA administration (data not shown).

The mGluII Receptor Antagonist LY341495 Attenuated the Elevations in ICSS Thresholds Associated with Spontaneous Nicotine Withdrawal. Mean (±S.E.M.) raw reward thresholds prior to mini-pump re-

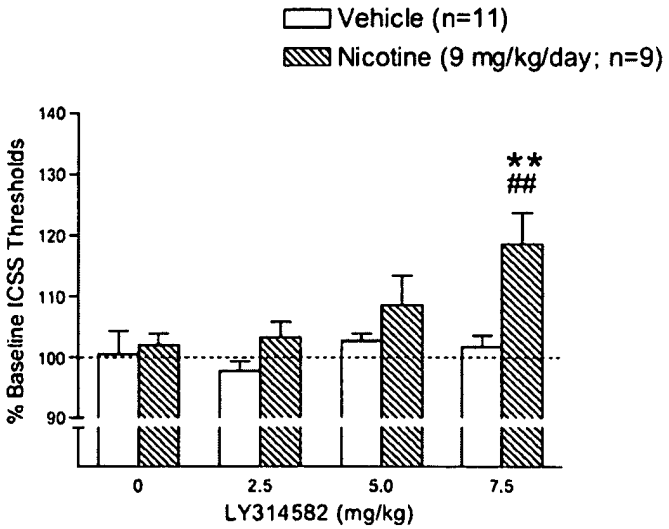

Fig. 2. Effects of LY314582 on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. **, $p < 0.01$, different from nicotine-treated rats after vehicle injection. ##, $p < 0.01$, different from control rats after injection with same dose of LY314582.

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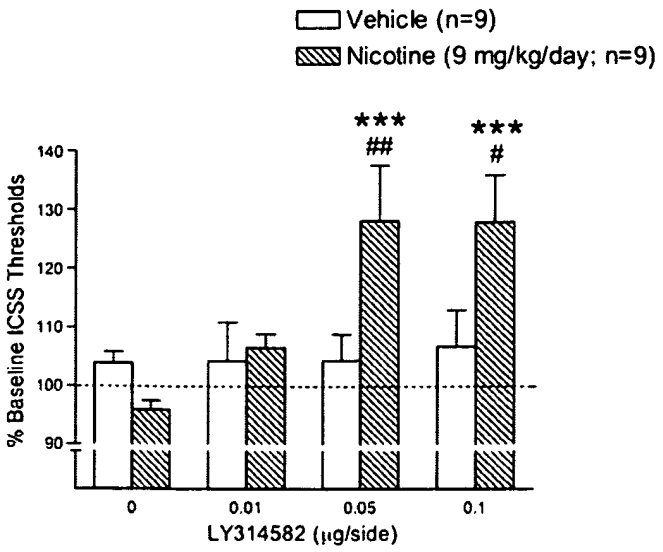

Fig. 3. Effects of intraventral tegmental area LY314582 on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. ***, $p < 0.001$, different from nicotine-treated rats after vehicle injection. ##, $p < 0.01$; #, $p < 0.05$, different from control rats after injection with same dose of LY314582.

moval for control and nicotine-treated rats were 105.9 ± 7.8 and 105.9 ± 11.0 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.38 ± 0.1 and 3.36 ± 0.13 μA, respectively. Withdrawal from chronic nicotine treatment produced robust ICSS threshold elevations compared with control rats [$F_{(1,27)} = 15.3$, $p < 0.001$] (Fig. 4A). Analysis of the significant group × dose × time interaction [$F_{(5,135)} = 3.3$, $p < 0.02$] revealed the following. Nicotine-treated rats injected with vehicle demonstrated robust reward threshold elevations that reached a peak 24 h after mini-pump removal (Fig. 4A). However, administration of LY341495 30 min before the 18-h time point significantly attenuated the elevations in reward thresholds in nicotine-withdrawing rats ($p < 0.001$) (Fig. 4A), without affecting thresholds in control rats (Fig. 4B). LY341495 had no effect on response latencies at any time point after injection [$F_{(1,27)} = 0.43$, N.S.] in nicotine-treated or control rats (data not shown).

The NMDA Receptor Antagonist Dizocilpine Lowered ICSS Thresholds Similarly in Nicotine-Treated and Control Rats. Mean (±S.E.M.) raw reward thresholds before treatment with the NMDA receptor antagonist dizocilpine for control and nicotine-treated rats were 88.9 ± 9.1 and 86.9 ± 3.2 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.32 ± 0.06 and 3.10 ± 0.06 μA, respectively. As can be seen in Fig. 5A, dizocilpine (MK-801; 0.01–0.2 mg/kg) lowered ICSS reward thresholds in nicotine-treated and control rats [$F_{(6,66)} = 7.5$, $p < 0.0001$], and there was no group × dose interaction [$F_{(6,66)} = 1.2$, N.S.]. Doses of dizocilpine ≥0.2 mg/kg caused disruption in performance in the ICSS paradigm in both groups such that rats no longer responded for self-stimulation, and therefore doses higher than 0.2 mg/kg were not tested. Furthermore, dizocilpine did not precipitate withdrawal-like elevations in reward thresholds in nicotine-treated rats at any dose tested. Dizocilpine significantly increased response latencies [$F_{(6,72)} = 2.9$, $p < 0.05$]. Post hoc analysis demonstrated that as the dose of dizocilpine increased, so too did response latency, particularly in control rats, suggesting that performance was increasingly impaired at higher doses of dizocilpine (Fig. 5B).

The mGlu5 Receptor Antagonist MPEP Elevated ICSS Thresholds Similarly in Nicotine-Treated and Control Rats. Mean (±S.E.M.) raw reward thresholds before treatment with low doses of the mGlu5 receptor antagonist MPEP for control and nicotine-treated rats were 118.9 ± 9.3 and 98.4 ± 8.9 μA, respectively. Mean (±S.E.M.) raw response latencies for the low-dose MPEP experiment for control and nicotine-treated rats were 3.34 ± 0.09 and 3.43 ±

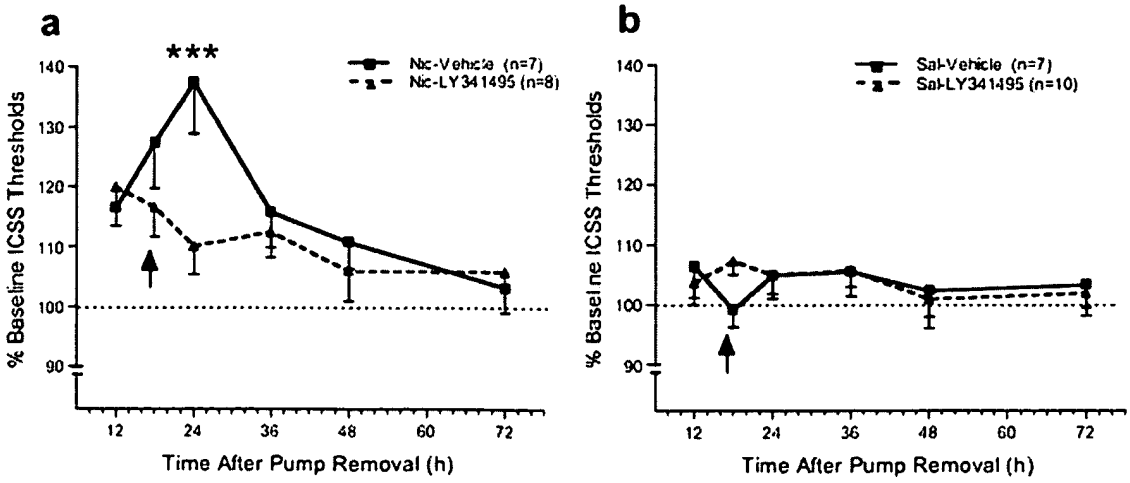

Fig. 4. Effects of LY341495 on the elevations in ICSS thresholds in rats undergoing spontaneous nicotine withdrawal. A, data are expressed as mean (±S.E.M.) percentage change from baseline thresholds in rats undergoing nicotine withdrawal. B, data are expressed as mean (±S.E.M.) percentage change from baseline thresholds in saline-treated control rats. ICSS thresholds were tested 12, 18, 24, 36, 48, and 72 h after surgical removal of osmotic mini-pumps delivering nicotine (Nic; A) or saline (Sal; B). Rats received a single injection of LY341495 (1 mg/kg) or vehicle (Veh) 30 min before the 18-h time point (indicated by black arrows). Solid line represents nicotine-withdrawing (A) or nicotine-naive (B) rats treated with vehicle 30 min before testing at the 18-h time point. Dashed line represents nicotine-withdrawing (A) or nicotine-naive (B) rats treated with LY341495 (1 mg/kg) 30 min before testing at the 18-h time point. ***, $p < 0.001$, different from rats undergoing nicotine withdrawal treated with vehicle 30 min before the 18-h time point.

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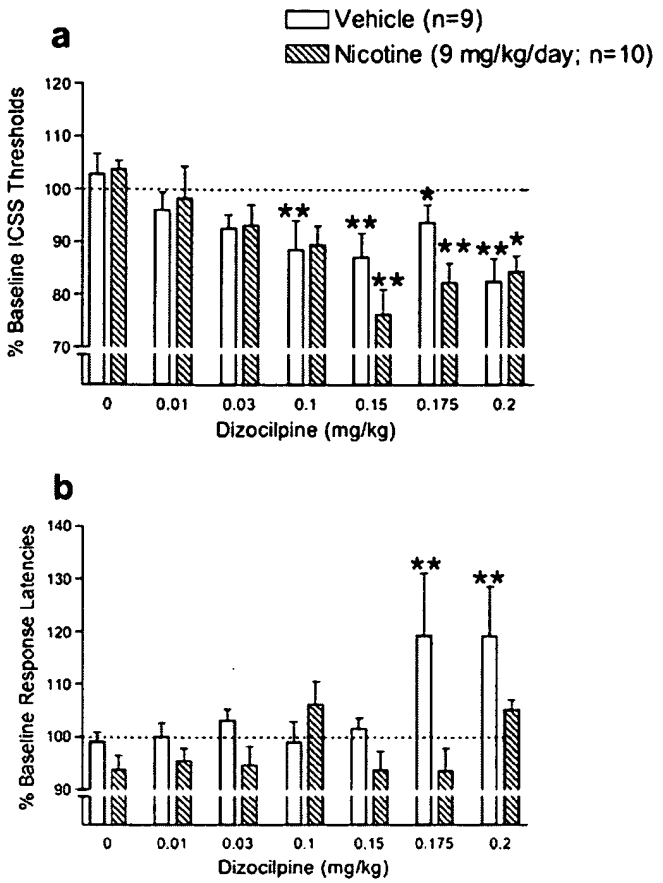

Fig. 5. Effects of dizocilpine (MK-801) on ICSS thresholds and response latencies in nicotine-treated and control rats. A, data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. B, data are expressed as mean (±S.E.M.) percentage change from baseline response latencies. *, $p < 0.05$; **, $p < 0.01$, different from corresponding vehicle- or nicotine-treated rats after vehicle injection.

0.12 s, respectively. Mean (±S.E.M.) raw reward thresholds before treatment with high doses of MPEP for control and nicotine-treated rats were 112.9 ± 8.9 and 109.5 ± 8.5 μA, respectively. Mean (±S.E.M.) raw response latencies for the high-dose MPEP experiment for control and nicotine-treated rats were 3.27 ± 0.19 and 3.14 ± 0.07 s, respectively. Low doses of MPEP (0.01–0.1 mg/kg) did not affect ICSS reward thresholds [$F_{(3,39)} = 2.3$, N.S.] or response latencies [$F_{(3,39)} = 0.4$, N.S.] in nicotine-treated or control rats (data not shown). Higher doses of MPEP (0.5–3 mg/kg) elevated ICSS thresholds in nicotine-treated and control rats [$F_{(4,96)} = 8.4$, $p < 0.0001$] (Fig. 6). However, MPEP elevated ICSS thresholds in both groups of rats by a similar magnitude (Fig. 6), and there was no group × dose interaction [$F_{(4,96)} = 0.7$, N.S.]. MPEP (0.5–3 mg/kg) had no effect on response latencies [$F_{(4,96)} = 1.4$, N.S.] in either group (data not shown).

The AMPA/Kainate Receptor Antagonist NBQX Precipitated Elevations in ICSS Thresholds in Nicotine-Treated but Not Control Rats. Mean (±S.E.M.) raw reward thresholds prior to treatment with the AMPA/kainate receptor antagonist for control and nicotine-treated rats were 98.9 ± 10.0 and 98.5 ± 11.8 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.21 ± 0.09 and 3.36 ± 0.15 μA, respectively. NBQX (0.01–1 mg/kg) significantly altered ICSS

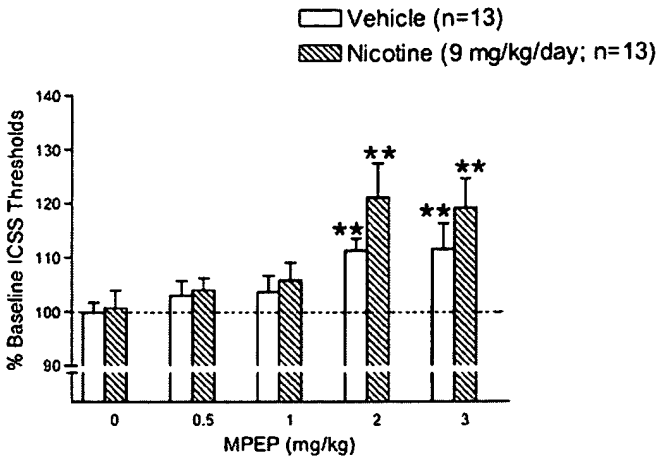

Fig. 6. Effects of MPEP on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. **, $p < 0.01$, different from corresponding vehicle- or nicotine-treated rats after vehicle injection.

thresholds in nicotine-treated but not control rats (Fig. 7). This effect was reflected in a statistically significant effect of group [$F_{(1,20)} = 10.82$, $p < 0.005$], a significant effect of dose [$F_{(7,140)} = 2.8$, $p < 0.01$], and a significant group × dose interaction [$F_{(7,140)} = 2.11$, $p < 0.05$]. Post hoc analysis revealed a bimodal action of NBQX on ICSS thresholds in nicotine-treated rats. Low doses of NBQX (0.025–0.1 mg/kg) elevated thresholds in nicotine-treated rats, whereas higher doses of NBQX (0.5–1 mg/kg) were less effective and did not significantly elevate thresholds compared with vehicle treatment (Fig. 7). NBQX had no effect on response latencies in nicotine-treated or control rats at any dose tested [$F_{(7,140)} = 0.31$, N.S.] (data not shown).

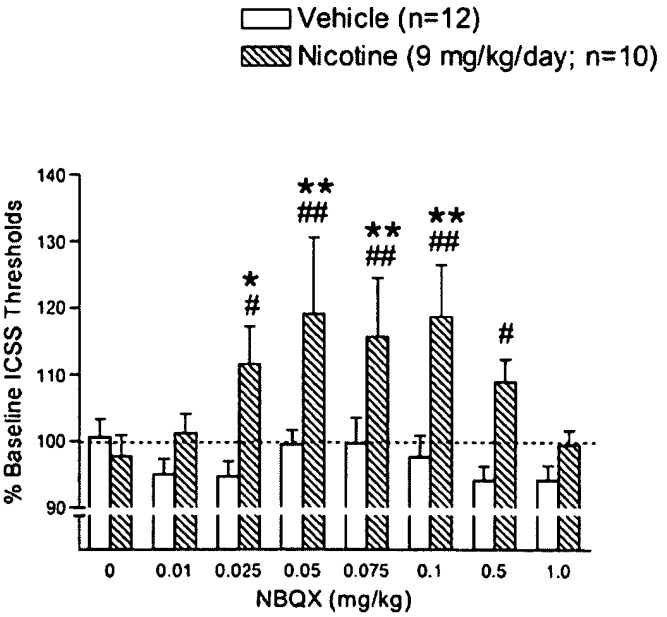

Fig. 7. Effects of NBQX on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. *, $p < 0.05$; **, $p < 0.01$, different from nicotine-treated rats after vehicle injection. #, $p < 0.05$; ##, $p < 0.01$, different from control rats after injection with same dose of NBQX.

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Discussion

Nicotine withdrawal precipitates an aversive abstinence syndrome in human smokers hypothesized to provide an important source of motivation contributing to the persistence of the smoking habit and relapse during abstinence (Kenny and Markou, 2001). The present data strongly suggest a role for group II metabotropic glutamate receptors in generating the reward deficits associated with nicotine withdrawal by demonstrating that activation of mGluII receptors precipitated ICSS threshold elevations in nicotine-dependent rats similar to those observed during spontaneous nicotine withdrawal. Furthermore, activation of mGluII receptors in the VTA also elevated thresholds in nicotine-dependent rats, providing further support for an important role of the VTA in mediating the actions of nicotine on reward pathways. Consistent with the above-mentioned information, blockade of mGluII receptors attenuated the reward deficits in rats undergoing spontaneous nicotine withdrawal. Previously, the mGluII receptor agonist LY354740 was shown to attenuate the increased auditory startle observed during spontaneous nicotine withdrawal (Helton et al., 1997). One possible explanation for these observations is that mGluII receptors located in different brain sites may differentially regulate various aspects of nicotine withdrawal.

Previously, the mGluII receptor agonist LY314582, which we found here to elevate reward thresholds in nicotine-dependent rats at doses ≤7.5 mg/kg, was shown to elevate ICSS thresholds in control rats at doses ≥10 mg/kg (Harrison et al., 2002). Therefore, the present observation that "subthreshold" doses of LY314582 precipitated withdrawal-like threshold elevations in nicotine-dependent but not control rats suggests that negative regulation of brain reward function by mGluII receptors was increased by prolonged nicotine treatment. One mechanism through which nicotine elicits its reinforcing effects is by increasing glutamatergic transmission in the VTA, thereby potentiating mesoaccumbens dopamine transmission (Schilstrom et al., 1998). Because mGluII receptors located in the VTA are presynaptic autoreceptors that decrease glutamate transmission (Bonci et al., 1997; Wigmore and Lacey, 1998), it is likely that the increased mGluII receptor sensitivity in the VTA observed in nicotine-treated rats occurred in response to prolonged activation of excitatory glutamate transmission by nicotine in this brain site, perhaps to counter this effect. Thus, during nicotine withdrawal when the stimulatory effects of nicotine on excitatory glutamate transmission were no longer present, increased mGluII receptor function would be expected to decrease glutamate transmission and thereby decrease the activity of this brain reward substrate. Recent electrophysiological studies are consistent with this hypothesis. For instance, chronic opiate treatment increased the inhibitory effects of mGluII receptor agonists on excitatory glutamate currents in VTA dopamine neurons (Manzoni and Williams, 1999), and in nucleus accumbens neurons (Martin et al., 1999). Nevertheless, it is possible mGluII receptors are also located on nonglutamatergic terminals (e.g., serotonergic and cholinergic neurons) and that activation of mGluII receptors precipitated nicotine withdrawal by decreasing the release of neurotransmitters other than glutamate. Indeed, nicotine withdrawal-induced threshold elevations were attenuated by coadministration of fluoxetine, a selective serotonin reuptake inhibitor, and 4-(2′-methoxyphenyl)-1-[2′(*N*-[2′-pyridinyl]-*p*-iodo-benzamido)ethyl]piperazine (P-MPPI), a serotonin-1A receptor antagonist, suggesting that decreased serotonergic transmission also contributes to the reward deficits associated with nicotine withdrawal (Harrison et al., 2001).

To further investigate a potential role of decreased glutamatergic transmission in the reward deficits associated with nicotine withdrawal, we examined whether antagonists at postsynaptic glutamate receptors precipitated withdrawal-like threshold elevations in nicotine-dependent rats similar to activation of mGluII receptors. At low doses the AMPA/kainate receptor antagonist NBQX precipitated threshold elevations in nicotine-treated but not controls rats. Under "normal" baseline conditions, AMPA receptors are the primary regulators of excitatory glutamate transmission throughout the mesoaccumbens reward pathway (Pennartz et al., 1990). Furthermore, AMPA receptor overexpression in the VTA increased, whereas AMPA receptor blockade decreased, the rewarding actions of drugs of abuse (Carlezon et al., 1997; Xi and Stein, 2002). These observations suggest that AMPA receptors positively modulate brain reward function. Conversely, AMPA receptor antagonists elicit an intrinsic rewarding action after VTA administration (David et al., 1998), suggesting that AMPA receptors may also negatively regulate brain reward function under baseline conditions. Indeed, AMPA receptors are located on dopamine and GABAergic neurons in the VTA (Wang and French, 1993, 1995), where they modulate mesoaccumbens dopamine transmission in an opposite manner. Therefore, it is possible that NBQX had no effects in control rats because it simultaneously blocked populations of AMPA/kainate receptors that positively and negatively regulate reward function. However, the sensitivity of nicotine-treated rats to NBQX suggests a scenario in which the development of nicotine dependence led to compensatory decreases in the number and/or function of those AMPA/kainate receptors that positively regulate brain reward function, perhaps to counter the prolonged stimulatory effects of nicotine on reward pathways. Consistent with this hypothesis, prolonged nicotine exposure decreased AMPA receptor immunoreactivity in the VTA and nucleus accumbens (Lee et al., 2002). Alternatively, it is possible that a "silent" population of AMPA/kainate receptors was recruited during prolonged nicotine exposure (Isaac et al., 1995), resulting in increased regulation of reward circuitries by AMPA/kainate receptors. Regardless of the mechanism, these data suggest that decreased glutamatergic transmission at AMPA/kainate receptors contributes to the threshold elevations observed in nicotine withdrawing rats.

There is considerable evidence that NMDA receptors play an important role in mediating the stimulatory effects of nicotine on mesoaccumbens dopamine transmission (Grillner and Svensson, 2000). Therefore, it might have been expected that prolonged nicotine treatment may have resulted in adaptations in the function/number of NMDA receptors such that their blockade precipitated withdrawal-like threshold elevations in nicotine-dependent rats but not controls similar to AMPA/Kainate receptor blockade. Nevertheless, this did not seem to be the case. Similar to previous reports (Carlezon and Wise, 1993), NMDA receptor blockade lowered thresholds in nicotine-dependent and control rats, indicating a rewarding action. At no dose tested did the NMDA receptor antagonist dizocilpine elevate thresholds in either nicotine-

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treated or control rats. Interestingly, dizocilpine tended to lower thresholds by a greater magnitude in nicotine-treated rats, suggesting they were slightly more sensitive to dizocilpine's reward-facilitating effects. Furthermore, higher doses of dizocilpine elevated response latencies in control but not nicotine-dependent rats, suggesting that prolonged nicotine treatment attenuated the performance-disrupting effects of dizocilpine. Nevertheless, based on the present data it is unlikely that decreased glutamatergic transmission at NMDA receptors contributes to the threshold elevations associated with nicotine withdrawal.

Recently, mGlu5 receptors, which are primarily located postsynaptically throughout the mesocorticolimbic system (Wigmore and Lacey, 1998), were shown to block the reinforcing effects of drugs of abuse, including nicotine (Chiamulera et al., 2001; Paterson et al., 2003). Therefore, we also investigated the role of mGlu5 receptors in nicotine withdrawal. At low doses, the mGlu5 receptor antagonist MPEP had no effect on ICSS thresholds, whereas higher doses elevated thresholds in nicotine-dependent and control rats (consistent with Harrison et al., 2002). Interestingly, MPEP tended to elevate thresholds by a greater magnitude in nicotine-dependent rats compared with control. However, because no dose of MPEP differentially elevated thresholds in nicotine-treated rats without also elevating thresholds similarly in control rats, these data indicate that mGlu5 receptors regulate baseline brain reward function in control and nicotine-treated rats, but are probably not involved in the threshold elevations associated with nicotine withdrawal.

Perhaps the most parsimonious explanation of the present observations is that prolonged, continuous nicotine exposure increased mGluII receptor function, and decreased AMPA/kainate-mediated glutamate transmission in reward circuitries, which contributed to the reward deficits observed during nicotine withdrawal. In contrast, recent investigations demonstrated that repeated, intermittent exposure to psychostimulants decreased mGluII function, and increased AMPA receptor transmission in reward circuitries (Giorgetti et al., 2001; Xi et al., 2002). Thus, it is possible that chronic nicotine and psychostimulant administration induce different alterations in glutamatergic transmission. Alternatively, this apparent discrepancy may be explained by the fact that the long-term behavioral effects of drugs of abuse are related to the dosing administration regimen (i.e., continuous or intermittent). Specifically, repeated intermittent exposure to addictive drugs can result in a progressive augmentation or "sensitization" in their behavioral effects (Pierce and Kalivas, 1997; Wolf, 1998). Conversely, more continuous exposure similar to that used in the present study, and similar to the pattern of prolonged nicotine exposure observed in smokers, engages counteradaptive "opponent processes" that decrease the acute behavioral effects of addictive drugs (i.e., "tolerance"), and leads to the expression of an aversive withdrawal syndrome upon cessation (Koob and Le Moal, 2001). It has been proposed that sensitization may be important in the early stages of drug addiction, when intake is intermittent, whereas tolerance and withdrawal may be more important in later stages of drug dependence, as drug intake progressively increases (Koob and Le Moal, 2001; Kenny et al., 2003). Based on the above-mentioned information, it is an interesting possibility that an initial increase, followed by a prolonged decrease in glutamatergic transmission, mediated by mGluII and AMPA/kainate receptors, may be involved in the initiation and maintenance of the drug-taking habit, respectively. Thus, it will be of interest to investigate whether other major drugs of abuse also increase the regulation of brain reward function by mGluII receptors.

In conclusion, the present data suggest that mGluII receptors play an important role in generating the reward deficits associated with nicotine withdrawal. Furthermore, it is likely that mGluII receptors generated these deficits, at least in part, by decreasing glutamate transmission at AMPA/kainate receptors. Thus, because the reward deficits associated with drug withdrawal are thought to play such a crucial role in drug addiction (Ahmed et al., 2002; Kenny et al., 2003), these data suggest that mGluII and AMPA/kainate glutamate receptors may prove to be useful therapeutic targets for the treatment of nicotine addiction.

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Address correspondence to: Dr. Athina Markou, Department of Neuropharmacology, CVN-7, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037. E-mail: amarkou@scripps.edu

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LY354740: a Metabotropic Glutamate Receptor Agonist which Ameliorates Symptoms of Nicotine Withdrawal in Rats

D. R. HELTON,* J. P. TIZZANO, J. A. MONN, D. D. SCHOEPP† and M. J. KALLMAN

Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN 46140, U.S.A.

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Summary—LY354740 is a conformationally constrained analog of glutamate with high selectivity and nanomolar agonist activity at Group II metabotropic glutamate receptors (mGluRs). This orally active compound is a new drug candidate which is being developed for the treatment of anxiety. In this study, LY354740 was investigated in a model of nicotine withdrawal using the acoustic startle reflex (sensorimotor reactivity) in rats. Nicotine (6 mg/kg/day) was administered for 12 days subcutaneously by osmotic minipumps. After 12 days the pumps were removed and the animals were allowed to go through spontaneous withdrawal. Cessation of chronic nicotine exposure led to increased startle responding for 4 days following withdrawal. Treatment with LY354740 (0.0001–0.1 mg/kg, i.p.; 0.03–3 mg/kg, oral) produced a dose-dependent attenuation of the enhanced auditory startle responding following withdrawal of nicotine with intraperitoneal and oral ED_{50} values of 0.003 mg/kg and 0.7 mg/kg, respectively. These effects were stereoselective since the (−)-enantiomer of LY354740, LY366563, was without effect in this model. LY354740 produced no changes in the sensorimotor reactivity of rats not exposed to nicotine at oral doses up to 10 mg/kg. These data support the functional role of mGluR agonists in nicotine withdrawal and indicate that LY354740 may be efficacious in reducing the symptoms associated with nicotine withdrawal during smoking cessation in humans. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Keywords—Metabotropic glutamate receptors (mGluRs), nicotine, withdrawal, sensorimotor reactivity, startle.

The excitatory neurotransmitter L-glutamate has been shown to activate ligand-gated cationic channels termed *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate (KA) receptors (ionotropic glutamate receptors; iGluRs) (Johnson and Ascher, 1987; Honore *et al.*, 1988; Lodge and Collingridge, 1991) and to regulate ion channels and enzymes producing second messengers via specific receptors coupled to G-proteins (Baskys, 1994). In contrast to ion channel-linked glutamate receptors (ionotropic) which facilitate fast synaptic transmission, metabotropic glutamate receptors (mGluRs) represent a heterogeneous family of receptor proteins that modulate synaptic function through coupling to multiple second messenger systems (Pin and Duvoisin, 1995). Metabotropic glutamate receptors are currently classified into three groups based on sequence homology, second messenger coupling and similar agonist pharmacology. Group I mGluRs include $mGluR_1$ and $mGluR_5$, which are coupled to phosphoinositide (PI) hydrolysis when expressed in non-neuronal cells. Group II mGluRs, which include $mGluR_2$ and $mGluR_3$, are negatively coupled to cyclic adenosine 3′,5′-monophosphate (cAMP) formation. Group III mGluRs are the most heterogeneous subgroup of mGluRs, and include $mGluR_4$, $mGluR_6$, $mGluR_7$ and $mGluR_8$. Group III mGluRs are also negatively coupled to cAMP formation when expressed in non-neuronal cells (Nakanishi, 1992; Schoepp, 1994; Pin and Duvoisin, 1995). The characterization of mGluRs in the central nervous system (CNS) represents a new area of therapeutic opportunity.

LY354740 [(+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate] (see Fig. 1) is a structural analog of glutamate which is highly selective for Group II metabotropic glutamate receptors. LY354740 has been shown to have no agonist or antagonist activity at Group I or Group III receptors up to 100 μM (Monn *et al.*, 1997; Schoepp *et al.*, 1997). Furthermore, LY354740 (up to

*Current address: Quintiles BRI, 1300 N. 17th Street, Arlington VA 22209, U.S.A.

†To whom correspondence should be addressed at: Neuroscience Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, U.S.A. Tel: 001 317 276-6316; Fax: 001 317 276-5546; e-mail: schoepp_darryle_d@lilly.com.

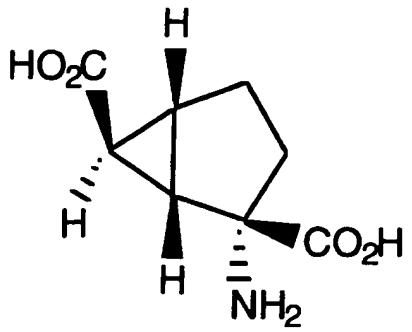

Fig. 1. Chemical structure of LY354740.

100 μM) does not displace radioligand binding at NMDA, AMPA or KA receptors (Monn *et al.*, 1997). The mGluR agonist effects of LY354740 are highly stereoselective, since LY366583, the (−) isomer of (+) LY354740, is >5000 times less active than LY354740, as shown by their EC_{50} values for decreasing forskolin-stimulated cAMP formation in rat brain slices (Monn *et al.*, 1997). LY354740, but not LY366583, has been shown to produce anxiolytic activity in the potentiated startle and elevated plus maze models of anxiety (Helton *et al.*, 1997; Monn *et al.*, 1997). As a result of these experimental findings, LY354740 is being developed for clinical investigation in the treatment of anxiety-related disorders.

Chronic administration of nicotine results in tolerance and dependence in both humans (Schachter, 1979; Shiffman and Phil, 1979; Henningfield *et al.*, 1995) and rodents (Clarke, 1987; Helton *et al.*, 1993). Cessation of chronic nicotine exposure in humans results in a number of withdrawal symptoms which include anxiety and irritability (Hughes and Hatsukami, 1992; Henningfield *et al.*, 1995). In rodents, however, signs of overt physical or behavioral withdrawal from chronic nicotine are limited. As such, there have been few robust, well accepted models for evaluating nicotine withdrawal in rodents (Emmett-Oglesby *et al.*, 1990). We have previously shown that cessation of chronic nicotine exposure in rats results in a robust increase in startle responding during the first 5 days following withdrawal, and re-administration of nicotine (0.4 mg/kg i.p.) greatly attentuates the enhanced startle response subsequent to nicotine withdrawal (Helton *et al.*, 1993). This enhanced sensorimotor responsiveness may reflect one or more of the withdrawal symptoms reported in man, and may provide a sensitive model for examining pharmacological intervention following the cessation of chronic nicotine exposure.

Metabotropic glutamate receptors can modulate both excitatory and inhibitory neuronal transmission by pre- and post-synaptic mechanisms. The role of glutamate in nicotine withdrawal is supported by an increasing number of studies reporting changes in glutamatergic function following both acute and chronic nicotine administration and/or withdrawal. For example, there have been reported interactions between the nicotinic cholinergic system and the glutamatergic system (Aizenman *et al.*, 1991; Vidal, 1994; Zhang *et al.*, 1994). Acute nicotine administration has been shown to enhance the release of glutamate through activation of nicotinic receptors located on presynaptic terminals and to facilitate evoked glutamate synaptic transmission (McGehee *et al.*, 1995; Gray *et al.*, 1996). However, the pharmacological characterization and understanding of the function of mGluRs in nicotine withdrawal have been limited due to a lack of potent, systemically active and selective pharmacological probes. In this study, the effects of the mGluR agonist, LY354740, were examined in rats undergoing withdrawal from chronic nicotine exposure. The effects of LY354740 were also compared to LY366583, which is the inactive (−) isomer of LY354740.

MATERIALS AND METHODS

Animals

Male Long–Evans rats (180–400 g) were obtained from Harlan Sprague–Dawley (Cumberland, IN, U.S.A.). All animals were acclimated for at least 3 days before testing. Animals were housed at 23 ± 2°C (relative humidity 30–70%) and given Purina Certified Rodent Chow and water *ad libitum*. The photoperiod was 12 hr light–12 hr dark, with dark onset at approximately 1800 hr. All experiments were performed in accordance with Eli Lilly and Company animal care and use policies.

Compounds

LY354740 monohydrate [(+)-2-aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate] and LY366563 [(−)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate] were synthesized as described by Monn *et al.* (1997). LY354740 and LY366563 were dissolved in a vehicle of purified water and neutralized with 5 N sodium hydroxide to a pH of approximately 7–8. Nicotine ditartrate (Research Biochemicals Inc., Natick, MA, U.S.A.) was dissolved in 0.9% saline. Food was removed at least 1 hr before testing.

Sensorimotor reactivity and nicotine withdrawal

For chronic nicotine administration, animals were anesthetized with isoflurane and Alzet osmotic mini-pumps (Model 2ML2; Alza Corporation, Vacaville, CA, U.S.A.) were implanted subcutaneously. Pumps were filled with nicotine ditartrate (6 mg/kg base per day) or vehicle (saline). Twelve days following implantation of pumps, rats were anesthetized with isoflurane and the pumps were removed. The auditory startle response (peak amplitude, V_{max}) of individual rats was recorded using San Diego Instruments startle chambers (San Diego, CA, U.S.A.). Startle sessions consisted of a 5-min adaptation period at a background noise level of 70 ± 2 dBA immediately followed by 25 presentations of auditory

stimuli (120 ± 3 dBA noise, 50 msec duration) presented at 8-sec intervals. Peak startle amplitudes were then averaged for all 25 presentations of stimuli for each session. All data are presented as overall session means. Baseline startle responding was evaluated prior to pump removal on day 12. Following chronic nicotine administration and removal of pumps, auditory startle responding was evaluated daily for 4 days at 24-hr intervals. Assessment began 24 hr after pump removal. LY354740 (0, 0.0001, 0.001, 0.01 or 0.1 mg/kg, i.p.; 0, 0.03, 0.3, 3 mg/kg, oral) was administered daily over the 4-day assessment period. Intraperitoneal LY354740 administration occurred 20 min prior to startle testing. Oral administration occurred 60 min prior to startle testing. The acute effects of LY354740 on startle responding was assessed in a separate experiment. In that experiment, startle responding was measured 60 min following an oral dose of 0, 1, 3 or 10 mg/kg LY354740. Startle data were analyzed using a one-way analysis of variance for each test day. When significant treatment effects were obtained, *post hoc* comparisons were made using a Tukey's studentized range (HSD) test. All statistical comparisons for nicotine withdrawal were made to the respective nicotine control group. In all analyses, two-tailed statistical tests were used and the level of significance was set at $p \leq 0.05$.

RESULTS

Sensorimotor reactivity—acute auditory startle

LY354740 had no significant effect on auditory startle responding in rats at oral doses up to 10 mg/kg (Table 1).

Sensorimotor reactivity and nicotine withdrawal

Intraperitoneal. Auditory startle responding was significantly increased (Tukey's HSD, $p \leq 0.05$) from 2 to 4 days following cessation of chronic nicotine exposure when compared to the response in control rats receiving vehicle (Fig. 2). Pretreatment with LY354740 (i.p.) produced an attenuation of the withdrawal-induced increase in startle responding with a significant attenuation at 0.001, 0.01 and 0.1 mg/kg on withdrawal days 2, 3 and 4 (Tukey's HSD, $p \leq 0.05$) when compared to nicotine controls (Fig. 2) (ED_{50} = 0.003 mg/kg LY354740; based on withdrawal day 2). The inactive (−) isomer of (+)LY354740, LY366583, did not attenuate the withdrawal-induced increase in startle responding at a dose of 0.1 mg/kg (i.p.) (Fig. 3).

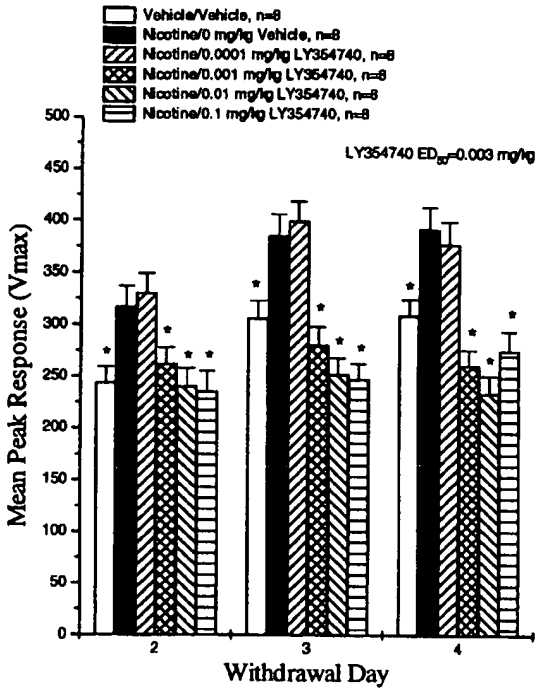

Fig. 2. Mean (±SE) peak startle amplitude (V_{max}). Auditory startle responding was evaluated on withdrawal days 2–4 at 24-hr intervals following removal of nicotine on day 12 (6 mg/kg/day, s.c.). Baseline startle responding was evaluated 24 hr before pump removal on treatment day 11 (data not shown). Vehicle or LY354740 (0, 0.0001, 0.001, 0.01 or 0.1 mg/kg, i.p.) was administered daily 20 min before startle evaluation. Vehicle represents rats administered saline for 12 days and then injected with water prior to startle assessment. Nicotine represents rats administered nicotine for 12 days and then injected with water or various doses of LY354740. *Significantly different from nicotine control, $p \leq 0.05$.

Table 1. Effect of orally administered LY354740 on sensorimotor reactivity as evaluated using auditory startle responding in Long-Evans rats

Compound	Dose (mg/kg, oral)	Startle response (μV)
Vehicle		251 ± 16
LY354740	1	212 ± 17
	3	221 ± 15
	10	300 ± 13

LY354740 was administered by oral gavage 60 min before testing. Values represent the mean (±SE) peak response (maximum startle amplitude) averaged over 25 trials. n = 10 rats/treatment group.

Oral. As shown in Fig. 4, LY354740 was administered orally, then tested in animals which had received either nicotine or saline vehicle by pump. In the baseline group (1 day prior to pump removal) there was no difference in startle response between animals receiving nicotine or the saline vehicle. LY354740 (0.03, 0.3 or 3 mg/kg oral, 60-min pretreatment) had no effect on baseline startle in the nicotine-treated animals. Auditory startle responding was significantly increased in nicotine-treated (pumps removed to induce withdrawal) animals (Tukey's HSD, $p \leq 0.05$) at withdrawal days 1, 2 and 3 when compared to the response in control rats (saline vehicle pumps removed) (Fig. 4). Pretreatment (60 min) with LY354740 (oral) during the withdrawal phase of the experiment produced an attenuation of the withdrawal-induced increase in startle responding, which was significant at 3 mg/kg on withdrawal days 1, 2 and 3 (Tukey's HSD,

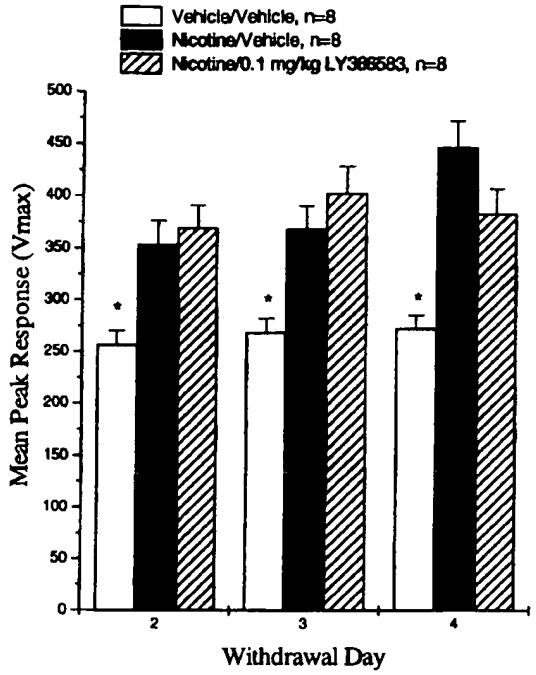

Fig. 3. Mean (±SE) peak startle amplitude (V_{max}). Auditory startle responding was evaluated on withdrawal days 2–4 at 24-hr intervals following removal of nicotine on day 12 (6 mg/kg/day, s.c.). Baseline startle responding was evaluated 24 hr before pump removal on treatment day 11 (data not shown). Vehicle or the inactive isomer (−)LY366583 (0 or 0.1 mg/kg, i.p.) was administered daily 20 min before startle evaluation. Vehicle represents rats administered saline for 12 days and then injected with water during the withdrawal period. Nicotine represents rats administered nicotine for 12 days and then injected with water or various doses of LY366583 during withdrawal. *Significantly different from nicotine control $p \leq 0.05$.

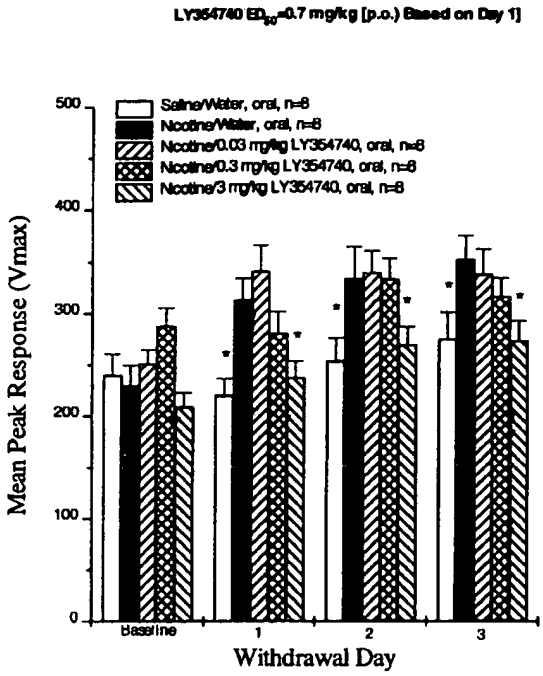

Fig. 4. Mean (±SE) peak startle amplitude (V_{max}). Auditory startle responding was evaluated on withdrawal days 1–3 at 24-hr intervals following removal of nicotine on day 12 (6 mg/kg/day, s.c.). Baseline startle responding was evaluated before pump removal on treatment day 12. Water or LY354740 (0.03, 0.3, 3 mg/kg, oral) was administered daily 60 min before startle evaluation during the withdrawal period. Saline/water represents rats administered vehicle for 12 days and then injected with water. Nicotine/water or LY354740 represents rats administered nicotine for 12 days and then injected with water or various doses of LY354740 during the withdrawal period. *Significantly different from nicotine $p \leq 0.05$.

$p \leq 0.05$) when compared to nicotine controls (Fig. 4) (ED_{50} = 0.7 mg/kg LY354740; based on withdrawal day 2).

DISCUSSION

The effects of LY354740 on the increased auditory startle responding seen in rats undergoing withdrawal from chronic administration of nicotine were evaluated to determine the potential use of LY354740 as an aid in nicotine withdrawal and/or smoking cessation. Increases in sensorimotor reactivity observed following withdrawal of nicotine were assessed by measuring the auditory startle reflex. Startle is a brainstem to spinal cord reflex which has proven useful for studying reflex alterations after pharmacological treatment, as nearly all defined neurotransmitter systems have been shown to interact in modulating the startle responding (Davis, 1987; Davis *et al.*, 1982). CNS hyperexcitability during nicotine withdrawal may be reflected in activation of common neural circuitry involved in the acoustic startle response. For example, cessation of chronic nicotine administration (6 or 10 mg/kg/day for 12 days, respectively) has been shown to produce significant increases in sensorimotor reactivity during the first 4–5 days of withdrawal (Helton *et al.*, 1993). Acute replacement of nicotine attenuates this increase in startle responding seen during cessation following chronic exposure.

LY354740 completely attenuated nicotine (6 mg/kg/day for 12 days, s.c.) withdrawal-enhanced startle responding following both intraperitoneal and oral administration with ED_{50}s of 0.003 and 0.7 mg/kg, respectively (Figs 2 and 4). The activity of LY354740 in this model was stereoselective since the inactive isomer, LY366563 (i.p.), did not attenuate the withdrawal-induced increase in sensorimotor reactivity (Fig. 3). LY354740 blocked potentiated startle responding at doses which produced no changes in acute basal startle (Table 1). In other work, we have found LY354740 to be devoid of benzodiazepine-like side-effects at anxiolytic or drug withdrawal suppressing doses. This includes effects on motor function as measured by spontaneous motor activity, neuromuscular coordination as measured

by rotorod, interaction with sedative hynotics such as hexobarbital, memory impairment as demonstrated in a passive avoidance procedure, or changes in convulsant threshold as demonstrated using electro-convulsive shock (Helton *et al.*, 1997). Other compounds which have been reported to work in this model include serotonin-1A antagonists (Rasmussen *et al.*, 1997) and the CCK-B antagonist LY288513 (Rasmussen *et al.*, 1996). These data indicate that the multiple neurotransmitter systems (i.e. serotonin, glutamate, neuropeptide) play a role in the nicotine-withdrawal phenomenon, and might represent novel approaches to treating smoking cessation in humans. Ultimately, human clinical trials will be needed to determine the relative roles of these different neurotransmitter mechanisms in smoking withdrawal in humans.

Interestingly, nicotine and LY354740, two different classes of pharmacological agents which suppress nicotine withdrawal behavior in rats, protect against conditions of glutamate over-excitation *in vitro* and *in vivo*. Nicotine protects cultured cortical (Akaike *et al.*, 1994) and striatal neurons (Marin *et al.*, 1994) from NMDA receptor-mediated excitotoxic injury *in vitro*. *In vivo*, nicotine also protects against the behavioral and neurotoxic effects of systemic kainic acid (Shytle *et al.*, 1996). Likewise, the group II mGluR agonist (2*S*,1′*R*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) protects cortical neurons from NMDA- and kainate-mediated neuronal degeneration *in vitro* (Bruno *et al.*, 1995; Buisson *et al.*, 1996), and intraventricular DCG-IV was a reported anticonvulsant and neuroprotectant against *in vivo* kainate in the rat (Miyamoto *et al.*, 1997). Thus, nicotine and group II mGluR agonists have in common the ability to protect against ionotropic glutamate receptor agonist-evoked seizures and neurodegeneration. However, nicotine and group II mGluR agonists have been shown to differentially affect the release of endogenous glutamate. DCG-IV or LY354740 block the enhanced release glutamate *in vitro* (East *et al.*, 1995; Di Iorio *et al.*, 1996) and *in vivo* (Battaglia *et al.*, 1997), whereas nicotine has been reported to enhance glutamate release *in vivo* (Toth, 1996). In the rat hippocampus, nicotine enhances glutamate transmission by a presynaptic action at mossy-fiber terminals (Gray *et al.*, 1996), whereas group II mGluR agonists decrease glutamate release at these same synapses (Kamiya *et al.*, 1996). How these acute effects of nicotine and group II mGluR receptor agonists relate to the ability of these compounds to suppress nicotine withdrawal is not clear.

It is possible that the actions of compounds such as LY354740, which acutely modulate glutamate excitation, may be altered in the nicotine-dependent animals. Glutamatergic neuronal transmission *per se* plays a role in neuronal adaptation to chronic nicotine (Stolerman *et al.*, 1995), since NMDA receptor antagonists have been shown to attenuate the development of tolerance to the locomotor depressant effects of nicotine (Shoaib *et al.*, 1994; Shoaib and Stolerman, 1996). Neuronal adaptive mechanisms which ensue following chronic nicotine have also been shown to alter the cellular actions of nicotine. For example, chronic nicotine has been shown to differentially alter nicotine-evoked release of certain neurotransmitters, increasing the ability of nicotine to induce the release of serotonin and dopamine, but decreasing nicotine-mediated acetylcholine release (Yu and Wecker, 1994). The effects of chronic nicotine on mGluR agonist or nicotine modulation of glutamate excitation (i.e. regulation of glutamate release) are not known.

In conclusion, LY354740 is a novel, systemically active pharmacological agent, representing a new therapeutic class for the potential treatment of human disease states. While LY354740 has been shown to have promising systemic activity in an animal model of nicotine withdrawal, much remains unexplored about this compound including identification of the brain site(s) and cellular mechanism of action responsible for its pharmacological activities. The relevance of the behavioral profile of LY354740 to the nicotine withdrawal state in humans remains to be tested.

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